Vol. 103, No. 3 December, 1952

THE
BIOLOGICAL BULLETIN

PUBLISHED BY THE MARINE BIOLOGICAL LABORATORY

THE ACTION OF DN-ASE ON CELL NUCLEI[1]

JAY BARTON II[2]

Department of Zoology, Columbia University, New York 27, New York

DNase (desoxyribonuclease) has been used as a cytochemical tool for some time because the unique localization of its substrate, DNA (desoxyribonucleic acid), calls for an investigation of its role in the structural organization of the nucleus. Mazia and Jaeger (1939) and Mazia (1941), using relatively impure nuclease preparations, demonstrated that the enzyme removed DNA from Drosophila and Chironomus chromosomes to the point of Feulgen-negativity without destroying the fibrous structure of the chromosomes. These basic observations have since been confirmed by Frolova (1941, 1944) and by Kaufmann and his co-workers, using highly purified enzyme preparations (Kaufmann, 1949; Kaufmann, Gay and McDonald, 1950). These workers have carefully examined the problems and possible errors of the cytochemical use of enzymes.

The fact of removal of DNA from chromosomes by DNase is unquestioned. Also unquestioned is the fact that the chromosome and nuclear structure is preserved after DNase action. The questions that the cytochemical experiments raise and cannot answer are: (1) is all the DNA homogeneous with respect to its removal by the enzyme, and (2) are any other constituents removed along with the DNA?

Such quantitative data may be obtained by analyzing the effects of DNase on isolated cell nuclei. Large amounts of material and straightforward chemical techniques may be used. An examination of how DNase can remove DNA from the nucleus, presumably by splitting inter-nucleotide bonds, can be expected to throw some light on the nature of the association between protein and nucleic acid in the intact nucleus.

Materials and Methods

Mammalian nuclei were isolated by the Mirsky-Pollister techniques using dilute citric acid (1946b). The nuclei were washed and suspended in buffer solution, either Sorenson's phosphate buffer or Michealis' veronal mixture.

Invertebrate sperm from various marine species were collected and washed with

[1] Part of this paper is taken from a dissertation presented to the Faculty of the Graduate School of the University of Missouri, Feb., 1951, in partial fulfillment of the requirements for the degree of Doctor of Philosophy. Support from the American Cancer Society, recommended by the Committee on Growth, National Research Council, through their grant to Dr. Daniel Mazia, is gratefully acknowledged.

[2] Atomic Energy Commission Predoctoral Fellow, 1949-50. Part of the data was collected during the tenure of this Fellowship.

filtered sea water. The sperm were then shaken with dilute citric acid and washed and suspended in buffer solution.

Carp sperm were collected from ripe fish and washed in a saline mixture (Mirsky and Pollister, 1946a). Turtle erythrocytes were washed in 0.18 *M* NaCl, hemolyzed in distilled water, gently blended in the Waring Blendor with dilute citric acid and washed until as free as possible from cell membranes. The reddish brown color of the nuclei indicated considerable contamination with hemoglobin.

Desoxyribonucleic acid was determined by the Dische diphenylamine method. The use of the Dische reaction directly on nuclear suspensions was justified by independent measurements using the Schneider technique for the extraction of nucleic acids from proteins prior to the diphenylamine test. When clear supernatants were measured, the Dische reaction was checked by independent measurements using UV absorption at 260 millimicrons in the Beckman spectrophotometer.

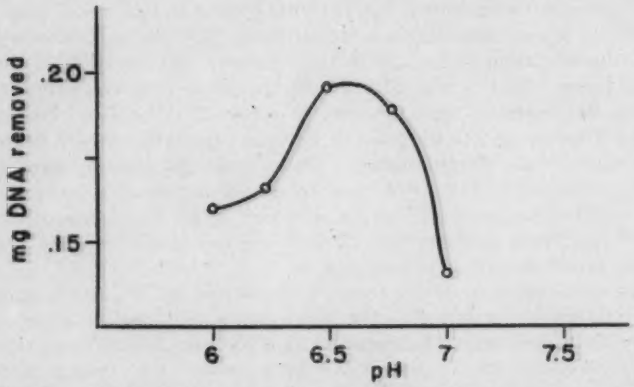


FIGURE 1. The pH optimum for removal of DNA from thymocyte nuclei by DNase. The points represent the amount of DNA removed in the first hour of digestion by the enzyme.

Amino nitrogen was measured by the Van Slyke manometric technique (1929). Hydrolysis of the sample for analysis was accomplished in 6 N sulfuric acid for 24 hours.

PROCEDURE

The nuclei were suspended in the buffer solution at pH 6.5. This was determined to be the optimum pH for the removal of DNA from the nucleus by DNase (Fig. 1). $MgCl_2$ was added as an activator to a final concentration of 0.01 *M*. Merthiolate (obtained through the courtesy of Eli Lilly Co.) was used in a final concentration of 1 : 10,000 to inhibit the growth of micro-organisms. Viscometric measurements of the activity of DNase in depolymerizing DNA in the presence of varying amounts of merthiolate were made by J. Neff (unpublished). No effect on DNase could be observed at the concentration of 1 : 10,000 used to inhibit bacterial growth. Crystalline DNase from the Worthington Biochemical Co. was then added. The mixture was incubated at constant temperature, 29.2° C. Samples were taken at intervals and centrifuged at about 4000 RCF. The clear supernatant,

free of nuclei, was analyzed for DNA. Control experiments were identical except that no enzyme was added.

Results

Release of DNA. As the digestion of the nuclei with DNase proceeds, DNA is released into the medium. The time course of the reaction is shown in Figure 2. The curve, representative in general of all experiments, describes the digestion of the sperm nuclei of the surf-clam, Mactra (Spisula). The most striking feature of the reaction is that not all the DNA can be removed from the nuclei even after prolonged digestion.

This effect, the failure of the DNase to remove all the DNA from the nuclei, has been found to occur in all forms tested. Table I shows the results of the enzymatic

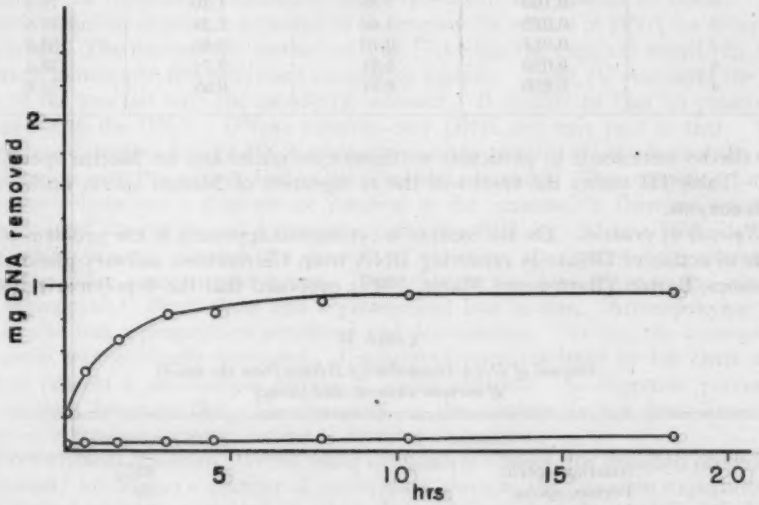


Figure 2. Time course of the removal of DNA from Mactra sperm nuclei by DNase. Sperm suspension in 0.05 *M* phosphate buffer, pH 6.5, 0.01 *M* with respect to $MgCl_2$. Enzyme conc. 4.0 microgm./ml. Total DNA = 2.5 mg./ml.

digestion of isolated thymocyte nuclei at various ratios of enzyme to substrate. The lowest value of 65.6 per cent DNA released is associated with the lowest enzyme-substrate ratio. The time curve of this particular reaction shows that the plateau had not been reached even after 35 hours of digestion. Table II summarizes the results of the digestion of nuclei from a wide variety of organisms. In all cases the amount of DNA left behind, *i.e.*, resistant to removal by DNase, is closely reproducible. The results of duplicate experiments on the same batch of nuclei ranged between two to three per cent. Variation among different batches of nuclei did not exceed this range. The differences in the amount of resistant DNA are associated with species characteristic differences and not to methodological variation.

That the DNA remaining with the nuclei is truly resistant to digestion and re-

moval can be demonstrated by the following observations. (1) Addition of fresh enzyme and/or activator to the reaction mixture does not result in additional release of DNA. (2) Washing the nuclei in fresh buffer solution does not remove any more DNA. (3) Washing and re-suspending the nuclei in fresh buffer, adding fresh enzyme and $MgCl_2$ does not result in further digestion. Such inten-

TABLE I

Release of DNA from beef thymocyte nuclei by DNase

Batch	Enzyme conc. mg./ml.	DNA in suspension mg./ml.	DNA released	
			Amount	Per cent
1	0.100	1.56	1.20	77.0
2	0.028	1.64	1.27	77.5
3	0.013	0.70	0.46	65.6
4	0.050	0.91	0.71	78.4
4	0.050	0.91	0.69	75.8

sive checks were made in particular on thymocyte nuclei and on Mactra sperm nuclei. Table III shows the results of the re-digestion of Mactra sperm nuclei with fresh enzyme.

Release of protein. On the basis of a cytological approach to the problem of the mode of action of DNase in removing DNA from Chironomus salivary-gland chromosomes, Barton (Barton and Mazia, 1949) proposed that the depolymerization of

TABLE II

Amount of DNA removable by DNase from the nuclei of various animals and tissues

Animal	DNA removable, %
Asterias, sperm	85%
Pecten, sperm	89
Mytilus, sperm	84
Arbacia, sperm	74
**Strongylocentrotus purpuratus*, sperm	80
**Strongylocentrotus franciscanus*, sperm	80
Mactra, sperm	40
Carp, sperm	82
Turtle, RBC (Caretta)	80
Rat, liver	70
Steer, thymus lymphocytes	77

* The data on Strongylocentrotus were kindly collected by Dr. Daniel Mazia, U. of California.

the DNA of the chromosomes led to the formation of low-molecular weight nucleoprotein complexes that were soluble at the ionic strength of the buffer solutions. Such a concept was in line with the behavior of artificial low-molecular weight DNA and basic protein complexes (Barton, 1948). Attempts were therefore made to determine the amount of protein released along with the DNA. On the basis

TABLE III

Re-digestion of Mactra sperm nuclei. After removal of 40% of DNA sperm re-suspended in 0.05 M PO_4 buffer, pH 6.5, 0.01 M $MgCl_2$. DNase conc. = 4 microgm./ml. Total DNA = 2.4 mg./ml.

Elapsed time	With enzyme DNA removed mg./ml.	Without enzyme DNA removed mg./ml.
1′	0.05	0.03
54′	0.05	0.03
7 hr. 46′	0.05	0.03

of Mirsky's data on isolated thymus chromosomes, the minimum amount of basic protein in the lymphocyte nucleus is about two-thirds the amount of DNA. The minimum amount of protein expected to accompany the release of DNA may then be calculated. The manometric method of Van Slyke has the requisite sensitivity and accuracy to measure this minimum amount of protein. Table IV compares the results of the analysis with the calculated amounts. It is obvious that no protein is released with the DNA. DNase removes only DNA and only part of that. The figures are corrected for the DNA nitrogen which is affected by the hydrolysis and which reacts with the nitrous acid in the Van Slyke method. The correction for 24 hours hydrolysis and a four-minute reaction in the manometric chamber amounts to about ⅕ of the purine and pyrimidine nitrogen—0.03 mg. N/mg. DNA.

Visible changes. During the process of digestion, the nuclei were examined under the phase contrast microscope. Two changes could be noted in the case of beef thymocytes. First, there was a pronounced loss in size. Accompanying the loss in size was a progressive wrinkling and deformation. Second, the contrast of the nuclei was gradually decreased. Undigested nuclei isolated by the citric acid method present a microscopic picture of sharp contrast. As digestion proceeds, the contrast becomes flat. The integrity of the nucleus is not lost, however. There is no tendency for the nucleus to dissolve or rupture.

Pretreatment of nuclei. When using mammalian tissues, the isolation procedure of necessity introduces a number of preliminary steps to the digestion experiments. These steps might be expected to affect the nucleic acid of the nuclei. Washing with citric acid is particularly suspect in this regard. By the use of invertebrate

TABLE IV

Determination of release of amino nitrogen accompanying removal of DNA from thymocyte nuclei by DNase

Digestion time hours	Amino N found* mg./ml.	Amino N expected** mg./ml.
0	+0.0068	0.007
3.3	−0.0050	0.055
13	+0.0087	0.075

* Difference between the blank and test analyses.
** Calculated on the basis 0.66 mg. protein per mg. DNA. Amino N is approximately 16 per cent of the protein.

sperm, it was thought possible to test the action of the citric acid. Sperm from Asterias, Mactra and Arbacia were washed in sea water and then suspended in the digestion mixture, as described above in the section on procedures, without any other pre-treatment. Use of both phosphate and veronal buffers in the digestion mixtures quickly resulted in the death of the sperm as indicated by the loss of motility. However, DNase was now completely ineffective in removing any of the DNA of the sperm. Treatment with dilute citric acid allowed DNase to remove part of the DNA. Duration of the treatment with citric acid did not appear to be significant. Nuclei washed once in one per cent citric, total exposure about 15 minutes, were as susceptible to DNase as were nuclei stored for several days in the citric acid.

Removal of the resistant fraction. In an attempt to determine why the cytochemical digestion studies had not disclosed the existence of so large an enzymatically resistant fraction of DNA, several techniques, inherent in the cytochemical method, were tested for their ability to remove the resistant fraction. Mactra sperm nuclei, previously subjected to digestion with DNase until 40 per cent of the DNA had been removed, were collected and fixed in Carnoy's fixative for 20 minutes. The fraction is stable to Carnoy's fixative. The nuclei were then suspended in 1 N HCl and heated to 60°–70° C. as in the Feulgen reaction. Three-minute hydrolysis removed a small amount of the DNA. Twenty-five minute hydrolysis removed all the DNA in the nuclei. Mazia and Jaeger (1939) had noted that the cytoplasm of the salivary gland cells gave a positive Feulgen reaction after treatment of the cells with DNase. It seems probable that the acid hydrolysis plus the sulfurous acid of the basic-fuchsin reagent in the Feulgen reaction caused the loss of the enzyme-resistant fraction.

Treatment of the nuclei so as to remove the basic protein renders them susceptible to complete digestion by DNase. Previously digested Mactra sperm nuclei containing 60 per cent of their original DNA were extracted for one-half hour with 0.2 N HCl. This treatment removes some or all of the basic protein of the nucleus. The nuclei were then washed and suspended in phosphate buffer, pH 6.6, $MgCl_2$ and 4 microgm./ml. of DNase added. The DNase can now remove all the remaining DNA in 8 hours. Extraction with buffer alone removed one-seventh of the DNA after 12 hours. These observations demonstrate that the resistant DNA fraction is only resistant to removal from the intact nucleus and is not intrinsically immune to hydrolysis by DNase.

Treatment of the partially digested nuclei with 2 *M* NaCl will also remove the remaining DNA along with protein. The extracted complex does not form a true solution and hence is not viscous. Preliminary studies of this complex were reported in 1951 (Barton).

Discussion

The most important question arising from this work is why all the DNA does not come out when subjected to digestion by DNase. There is, of course, no *a priori* reason why breaking internucleotide bonds should result in the removal of the hydrolyzed fragments. The effectiveness or lack of effectiveness of DNase in removing DNA must be related to the nature of the linkage between DNA and the protein components of the nucleus or to the spatial orientation of the DNA in the nucleus.

Various hypotheses for the failure of DNase to remove all the DNA may be suggested. (1) The enzyme has been inhibited by products of the reaction. This proposal may be rejected on the basis of the redigestion experiments with fresh enzyme. Washing of the nuclei should have removed any inhibitor. (2) All the DNA may have been digested, but the fragments have complexed with or adsorbed on the proteins of the nucleus. In such a case, an equilibrium should exist beween DNA complexed and DNA in solution. Washing should shift the equilibrium and remove complexed DNA fragments. This is not observed.

Can the DNA remaining with the nuclei be identified with any DNA fraction previously reported in the literature? Zamenhof and Chargaff (1949) reported the discovery of an enzymatically resistant fraction of beef thymus DNA. Similar results have been reported by Overend and Webb (1950). It is clear that the fraction described in this paper is enzymatically resistant to removal, but the amount of the fraction is considerably larger than the "core" reported by Chargaff and by Overend. The core reported in thymus DNA amounted to no more than 7 per cent of the total DNA. Chargaff's analysis of the core showed that it contained a higher ratio of purines to pyrimidines than did the intact DNA. The remainder of the DNA, *i.e.*, the dialyzable portion of the enzymatic digest, showed a higher ratio of pyrimidines to purines than did intact DNA. In the experiments reported here on the removal of DNA from nuclei, analyses of the DNA released by the enzyme were made by UV absorption, which measures both purine and pyrimidine bases; these checked closely with analyses made by the diphenylamine reaction which gives a measure only of purine-containing nucleotides. Pending a quantitative analysis of the bases in the removable and resistant fractions, the conclusion is reached that the resistant fraction does not correspond to a core resistant to the enzyme by virtue of a high purine nucleotide composition. The ability of DNase to remove the fraction following extraction with 0.2 N HCl supports this conclusion.

Mirsky (1947) has reported that the isolated "chromosomes" of thymocyte nuclei contain two fractions of DNA, one removable with strong NaCl and a second residual fraction, only a few per cent of the total, associated with the residual chromosome. The amount of the discrepancy with an enzymatically resistant fraction of 23 per cent reported here is too great to suppose an identity between the two.

The evidence at present points to the conclusion that the fraction of DNA resistant to removal by DNase is a spatially separate component of the total DNA. It is not intrinsically resistant to DNase, but only so because of its location in the nucleus. Removal of basic protein by treatment with 0.2 N HCl allows the enzyme to act. The block to complete removal of the DNA by DNase may be interpreted in two ways. (1) First, the basic protein may take part in an actual masking of the substrate. Thus, one portion of the DNA, the easily removable one, would be "exterior" to the remainder of the nucleoprotein complex; the second portion of the DNA, the resistant one, would be "interior," surrounded by the basic protein. (2) The second DNA component may be bound to protein of the nucleus in such a manner as to be immune to attack by DNase. Treatment with 0.2 N HCl would not only extract the basic protein but would dissociate or alter the complex so that the DNase could now act. Preliminary studies of the resistant DNA complex, removed by extraction with strong NaCl solutions as described above, indicate that the

nucleoprotein does not behave in a typical manner. In particular, it is not dissociated into DNA and protein by media of high ionic strength.

A choice between these possibilities must await detailed examination of the resistant fraction.

No explanation can be offered for the variation of the amount of resistant DNA among the various species studied except to point out that the amounts fall into two classes: one centering around 80 per cent, the other (Mactra) at 40 per cent. One feature of the chemistry of the nuclei of the species that also separates them into the same two classes is the nature of the basic protein contained in the nuclei. In all the species except Mactra, the basic protein is of the histone type containing approximately 20–25 per cent basic amino acids. Mactra, on the other hand, contains in its sperm nucleus a basic protein more closely resembling protamine in its solubility properties and would thus be expected to have a considerably higher proportion of basic amino acids (Barton, 1951).

The action of dilute citric acid in the pretreatment of the nuclei for digestion is not clear. The citric acid may affect the nuclei in at least two ways. (1) It may complex with calcium of the nuclei, thus altering the state of DNA and protein within the nuclei. (2) It may alter the nuclear membrane profoundly. A "toughening" effect of dilute acids on nuclear membranes was first observed by Crossmon (1937). Further study with other calcium complexing agents and with other means of killing are in progress.

The work on marine forms was made possible by the generosity of the Lalor Foundation through Lalor Foundation Fellowships during the summers of 1951 and 1952 at the Marine Biological Laboratory, Woods Hole, Mass.

Summary and Conclusions

1. The digestion of isolated nuclei by DNase has led to the following conclusions: (a) not all the DNA can be removed by the enzyme; (b) no protein is released with the DNA; (c) treatment of the residue with 0.2 N HCl removes the basic protein; it does not remove the DNA, but renders the resistant fraction susceptible to digestion with DNase.

2. The resistant fraction of the DNA cannot be correlated satisfactorily with any previously reported DNA fractions. The resistance to removal can be interpreted as due to the spatial orientation of the DNA within the nucleus or to the unique mode of combination with protein of the nucleus.

LITERATURE CITED

Barton, J., 1948. Protamine precipitation as a means of determining DNase activity. Master's thesis, University of Missouri, Columbia, Missouri.

Barton, J., and D. Mazia, 1949. Specificity of desoxyribonucleases and their cytochemical application. *Biol. Bull.*, **97**: 267.

Barton, J., 1951. A new DNA fraction of the cell nucleus. *Biol. Bull.*, **101**: 236.

Crossmon, G., 1937. The isolation of muscle nuclei. *Science*, **85**: 250.

Frolova, S. L., 1941. Chromosome structure after the removal of thymonucleic acid by action of enzymes. *C. R. Acad. Sci. U.R.S.S.*, **30**: 459–462.

Frolova, S. L., 1944. Study of fine chromosome structure under enzyme treatment. *J. Heredity*, **35**: 235–246.

KAUFMANN, B. P., 1949. Enzymatic studies of cellular organization. *Science*, **109**: 443–444.

KAUFMANN, B. P., H. GAY AND M. R. MCDONALD, 1949. Localization of cellular proteins by enzymatic hydrolysis. *Cold Spring Harbor Symp. Quant. Biol.*, **14**: 85–90.

KAUFMANN, B. P., H. GAY AND M. R. MCDONALD, 1950. Analysis of cell structure by digestion with enzymes. *Genetics*, **35**: 117–118.

MAZIA, D., AND L. JAEGER, 1939. Nuclease action, protease action and histochemical tests on salivary chromosomes of Drosophila. *Proc. Nat. Acad. Sci.*, **25**: 456–461.

MAZIA, D., 1941. Enzyme studies on chromosomes. *Cold Spring Harbor Symp. Quant. Biol.*, **9**: 40–46.

MIRSKY, A. E., 1947. Chemical properties of isolated chromosomes. *Cold Spring Harbor Symp. Quant. Biol.*, **12**: 143–146.

MIRSKY, A. E., AND A. W. POLLISTER, 1946a. The nucleoprotamine of trout sperm. *J. Gen. Physiol.*, **30**: 101–116.

MIRSKY, A. E., AND A. W. POLLISTER, 1946b. Chromosin, a desoxyribose nucleoprotein complex of the cell nucleus. *J. Gen. Physiol.*, **30**: 117–148.

OVEREND, W. G., AND M. WEBB, 1950. Deoxypentose nucleic acids. Part I. The enzymic degradation of sodium deoxyribonucleate by deoxyribonuclease. *J. Chem. Soc.* (London), 2746–2754.

VAN SLYKE, D. D., 1929. Manometric determination of primary amino nitrogen and its application to blood analysis. *J. Biol. Chem.*, **83**: 425–447.

ZAMENHOF, S., AND E. CHARGAFF, 1949. Evidence of the existence of a core in DNA. *J. Biol. Chem.*, **178**: 531–532.

THE AGGLUTINATION OF FISH ERYTHROCYTES BY NORMAL HUMAN SERA

JOHN E. CUSHING AND LUCIAN SPRAGUE[1]

Dept. of Biological Sciences, Santa Barbara College, Santa Barbara, California

The discovery that normal human sera strongly agglutinate the erythrocytes of some species of fish led the authors to investigate this phenomenon systematically. This study represents part of a general program (Cushing, 1952a, 1952b) intended to develop serological techniques that might be useful in making racial and evolutionary studies on fish similar to those being made on man (Race and Sanger, 1950) and such other animals as doves (Irwin, 1947) and cattle (Stormont, Owen and Irwin, 1950).

Although Nuttall (1904) included fish in his classical work on the serological differentiation of animal bloods, relatively little has since been done with the serology of this group of vertebrates. The references known to these authors are listed in the bibliography or may be found by consulting the papers given. These include work by the following: Streng, 1938; Cushing, 1942, 1945a, 1945b; Gemeroy, 1943; Jonsson, 1944; Tyler and Metz, 1945; Tyler, 1946; and Grubb, 1949.

In addition, attention is called to the work of Cotterman (1944) who used normal human serum to demonstrate differences in mice of the genus *Peromyscus* and to that of Tamm (1952) demonstrating species differences in the viral agglutination of fish erythrocytes.

In order to establish a point of departure for comparative study, two species of marine fish were selected, the shiner sea-perch, *Cymatogaster aggregata* (Gibbons), a member of the viviparous perches (Embiotocidae), and the kingfish, *Genyonemus lineatus* (Ayres), a croaker (Sciaenidae). These were chosen primarily because their red blood cells were strongly agglutinated by human sera and because of the ease with which they could be captured locally and kept alive in the laboratory.

Materials and Methods

The physiological saline used was prepared by diluting filtered sea water with an equal volume of distilled water. This gave a final concentration of approximately 1.5% sodium chloride. The use of sea water avoided the possibility of hemolysis induced in the cells of some species of fish by some artificially prepared sodium chloride solutions (Ball, 1933). The agglutination of human erythrocytes was not affected by the use of this saline.

Fish erythrocytes were obtained by heart puncture. Blood was drawn into syringes containing an approximately equal volume of heparin solution. This solution had a concentration of 50 mg. of heparin for every 10 ml. of physiological saline. This preparation was found to be satisfactory for most of our work, but further re-

[1] Present address: Division of Genetics, University of California, Davis, California.

search on the usefulness of other concentrations of heparin and on other kinds of anti-coagulants is desirable.

Erythrocytes were washed free of serum proteins in physiological saline and finally suspended in a concentration approximating two per cent cells.

The human sera used in these studies were obtained either from local individuals, in which case their complement was heat-inactivated before use, or in the case of blood typing sera, from the Cutter Laboratories, Berkeley. The particular sera used in an experiment are referred to in the legends of the tables presenting the data for that experiment. Sera were kept frozen when not being used, inasmuch as repeated freezing and thawing were not observed to have any effect upon the agglutinins being studied.

Slide agglutination tests were used to determine the presence or absence of the various antigens and antibodies studied. These tests were made at room temperature by placing single drops of the appropriate cell suspensions and sera together and mixing these by hand rotation of the slide. Readings were made by eye at the end of 15 minutes without the aid of a microscope and checked at 30 minutes. Drying was prevented by placing Petri dish covers over the slides.

Absorptions of sera by erythrocytes were made by placing 0.1 ml. of packed cells in small test tubes with 1.0 ml. of serum and either rotating these mixtures at a nearly horizontal angle by attaching the tubes to a disc turned on a stirring motor, or by placing the tubes in a horizontal position and shaking them through their long axes on a Yankee-Kahn Shaker. In this later procedure, a small glass bead was added to each tube. The time of absorption varied in individual cases from 5 to 20 minutes. In general it was found that a five-minute absorption on the shaker was sufficient to obtain suitable preparations.

These preparations were obtained with a view of decreasing the titers of the antibodies being absorbed relative to those not being absorbed to a point where the slide test gave negative readings of the absorbed antibody while showing that the titers of unabsorbed antibody remained the same. While the slide test was entirely adequate for the studies reported here, it has been suggested that the test tube method (Schiff and Boyd, 1942; pp. 31–33), enhanced by centrifugation, may prove to be a faster and more sensitive test for future work.

Care was taken to insure that all the sera used were sterile and therefore did not contain agglutinins produced by bacteria.

Results

The antigens described below represent the minimum number of antigenic differences that can be detected between *Cymatogaster* and *Genyonemus* cells. The possibility exists that a single antigenic entity may be resolved into two or more components with the discovery of suitable antibodies. The F-3 and F-4 antigenic components described below were thus resolved.

The results of studies of the sera of several individuals, which were collected at random, indicate that the titers of red cell agglutinins for fish and humans vary independently. The indicated titers (Table I) should be taken as reasonable approximations only, inasmuch as it is possible that individual variations in red cells might influence the relative strength of the reactions to some extent (Wiener, 1943; p. 20). The agglutinins involved are stable to heating at 56° C. for 25 minutes. That dif-

TABLE I

Agglutination of fish and human erythrocytes by normal human sera

Serum from human type	Cells from	Serum dilutions				
		1/4	1/8	1/16	1/32	1/64
AB	*Cymatogaster*	3+	+	±	0	0
	Genyonemus	0	0	0	0	0
AB	*C.*	+	±	0	0	0
	G.	0	0	0	0	0
A	*C.*	2+	+	0	0	0
	G.	±	0	0	0	0
	B (Human)	±	0	0	0	0
A	*C.*	4+	4+	3+	2+	+
	G.	±	0	0	0	0
	B H.	±	±	0	0	0
B	*C.*	4+	3+	3+	±	0
	G.	2+	±	0	0	0
	A H.	+	0	0	0	0
B	*C.*	2+	+	0	0	0
	G.	0	0	0	0	0
	A H.	2+	±	0	0	0
O	*C.*	4+	4+	3+	2+	0
	G.	3+	+	0	0	0
	A H.	2+	+	±	0	0
	B H.	3+	2+	+	0	0
O	*C.*	3+	3+	+	±	0
	G.	+	0	0	0	0
	A H.	+	±	0	0	0
	B H.	+	+	±	0	0
O	*C.*	4+	3+	3+	2+	2+
	G.	2+	±	0	0	0
	A H.	3+	3+	+	0	0
	B H.	3+	+	+	0	0

The reactions of several human sera with fish and human erythrocytes are shown. All sera were from a single collection, twenty-four hours old, and were heat-inactivated at 56° C. for 25 minutes before using. Agglutination tests were run as described in the text. Degree of agglutination, estimated visually in terms of the number of cells agglutinated, is described as follows: 4+ = essentially all; 3+ = three-fourths; 2+ = one-half; + = one-fourth; ± = definite agglutination but less than one-fourth.

ferent agglutinins from the anti-A anti-B agglutinins may be involved in the reactions is suggested by the fact that the fish and the human red cell titers vary independently. Further evidence for this conclusion is obtained by the absorptions indicated in Table II. It can be seen from this table that since *Cymatogaster* cell absorption of anti-A plus anti-B sera fails to remove either anti-A or anti-B from the

pooled sera, *Cymatogaster* cells must possess at least one antigen distinct from the A and B antigens on human cells. This is at once apparent when the sera of type AB individuals are studied since these individuals lack detectable anti-A or anti-B bodies in their sera but show titers against fish red cells.

TABLE II

Differentiation of an antigen on Cymatogaster erythrocytes

Unabsorbed sera	Cells			Sera absorbed with these cells	Cells		
	Human A	Human B	*Cymatogaster*		Human A	Human B	*Cymatogaster*
Anti-A	4+	0	4+	Human A	0	0	4+
Anti-B	0	4+	4+	Human B	0	0	4+
Anti-A+B	4+	4+	4+	*Cymatogaster*	4+	4+	0

The antisera were commercial preparations derived from pooled human sera. These were used in a final dilution of 1 in 2 from that of the original preparation as received in this laboratory. The anti-A+B serum was prepared by mixing equal volumes of the anti-A and anti-B and was used without further dilution.

The reactions of *Genyonemus* cells with human typing serum are more complex (Table III). *Genyonemus* cells carry an antigenic component that is different from that of *Cymatogaster* for it resembles the human B antigen in its reactions with anti-B serum. Further evidence for this point is the fact that *Genyonemus* cells are much more rapidly and strongly agglutinated by anti-B serum than by anti-A serum.

TABLE III

Demonstration of an antigen on Genyonemus cells that is serologically related to the human B antigen

Cells	Unabsorbed serum			Absorbed with *Genyonemus* cells			Absorbed with B cells		
	1/4	1/8	1/16	1/4	1/8	1/16	1/4	1/8	1/16
Human B	4+	3+	+	+	0	0	0	0	0
Cymatogaster	4+	3+	+	4+	3+	+	4+	3+	+
Genyonemus	3+	2+	0	0	0	0	0	0	0

The serum used was obtained from a type A human and was selected because it not only contained human anti-B and *Genyonemus* red cell agglutinins, but *Cymatogaster* red cell agglutinins as well.

Genyonemus cells react as strongly with anti-A typing serum after absorption of the anti-A antibodies as before (Table IV). The *Genyonemus* cells have at least one antigen other than the B antigen on their cells. This antigen is not related to human A. This point is confirmed by the occurrence of a human AB serum that agglutinates *Genyonemus* and *Cymatogaster* cells, but not human cells of type A or B (Table V).

It is therefore concluded that *Genyonemus* erythrocytes possess one antigen very similar to the human B antigen, and a second antigen distinct from either the human A or B antigen. *Cymatogaster* can be shown to possess one antigen distinct from the human A or B antigens.

For convenience in further discussion the two *Genyonemus* antigenic components will be numbered F-1 (the B-like antigen) and F-2 (the not-B antigen).

TABLE IV

Differentiation of Genyonemus and human A antigens

Cells	Dilutions of unabsorbed anti-A			Dilutions of anti-A absorbed with human A cells		
	1/1	1/2	1/4	1/1	1/2	1/4
Human A	4+	4+	3+	0	0	0
Cymatogaster	4+	3+	±	4+	3+	±
Genyonemus	2+	±	0	2+	±	0

The antiserum used was a commercial preparation of pooled human sera and was diluted after absorption.

The *Cymatogaster* antigen will be numbered F-3. That it is distinct from F-1 and F-2 will be shown below. The letter F is used here to denote that these are antigens found on fish cells, and to give distinctiveness to the numbers used.

The differentiation of F-2 and F-3 is based upon several facts. First, the anti-B serum absorbed with *Genyonemus* cells still retained an unimpaired ability to agglutinate *Cymatogaster* cells (Table III) which they would not have done had F-2 and

TABLE V

Differentiation of an antigen on Genyonemus cells

Cells	Dilutions of unabsorbed serum				Dilutions of serum absorbed with *Genyonemus* cells			
	1/2	1/4	1/8	1/16	1/2	1/4	1/8	1/16
Cymatogaster	4+	4+	+	0	4+	4+	+	0
Genyonemus	4+	+	0	0	0	0	0	0

The antiserum used was from an AB individual selected because it agglutinated both *Cymatogaster* and *Genyonemus* cells, but failed to agglutinate human A, B or sheep red cells. The serum was diluted after absorption.

F-3 been alike antigenically. Second, the absorption with *Genyonemus* cells of the AB serum capable of agglutinating both *Genyonemus* and *Cymatogaster* cells left the *Cymatogaster* agglutinins intact (Table V).

Additional evidence shows that *Cymatogaster* and *Genyonemus* cells each have at least one antigen peculiar to themselves, although not necessarily F-2 and F-3. A rabbit anti-*Cymatogaster* serum that agglutinated *Cymatogaster* cells in dilutions

greater than 1 in 600 failed to agglutinate *Genyonemus* cells in any dilution. Also a rabbit anti-yellowfin tuna serum strongly agglutinated *Cymatogaster* cells and only weakly agglutinated *Genyonemus*. The reverse was true of an anti-skipjack serum that did not agglutinate human B cells.

Once distinguished, the F-3 (*Cymatogaster*) antigen could be further resolved into two separate antigenic components on the basis of the following observations. Sera of human AB individuals were found which agglutinated *Cymatogaster* but not sheep cells. The designation F-3 is retained for the antigen involved in this agglutination reaction.

In addition, anti-sheep cell sera were found to agglutinate strongly *Cymatogaster* cells but not those of *Genyonemus*. Absorption of rabbit anti-sheep cell serum with *Cymatogaster* cells left agglutinins for sheep cells only, but sheep cells removed both sheep and *Cymatogaster* agglutinins from this serum (unabsorbed sheep cell titer 1 in 1000, unabsorbed *Cymatogaster* titer 1 in 64). These reactions therefore reveal a second antigen on *Cymatogaster* cells, designated as F-4.

These observations, together with the fact that sheep cells are hemolysed by the action of heat-inactivated rabbit anti-*Cymatogaster* serum (titer of hemolysin = 1 in 40) in the presence of guinea pig complement, suggest that F-4 is one of the so-called Forssman antigens (Boyd, 1947).

So far, no individual antigenic variations have been detected within the two species. However, a persistent search for these was not made.

Discussion

We find that normal human sera contain at least three agglutinins that are specific for antigenic components on the erythrocytes of *Genyonemus* and *Cymatogaster*. One of these is the anti-B agglutinin, while the other two represent antibodies not related to the classical isoantibodies. Whether these antibodies are genetically determined, or are the result of immune responses to infection (Wiener, 1951), remains to be found. The three antibodies serve to identify two antigenic components on *Genyonemus* red cells, one of which is similar to the human B antigen, and one antigenic component on *Cymatogaster*.

In addition, rabbit anti-serum against sheep erythrocytes has been used to demonstrate a second antigenic component on *Cymatogaster* erythrocytes. This component resembles the Forssman antigens in its properties.

The term antigenic component is used to differentiate the two serologically distinct entities of each species for the reason that available evidence cannot differentiate between two alternative hypotheses: (1) that each serologically recognizable component of a given fish is actually a distinct and separate antigen; (2) that each serologically recognizable component of a given fish represents a difference in the reactions of two kinds of antibodies with a single antigen on the cells of that fish.

In other words, unless the antigenic components can be separated genetically (or otherwise) it is possible that *Cymatogaster* and *Genyonemus* may differ only in a minimum of two antigens rather than four. The problem involved is similar to that encountered in the study of some of the antigens in cattle (Stormont, Owen and Irwin, 1950), although the situation in cattle is, of course, much more complex, involving genetic as well as serological data.

No individual variations in the occurrence of these antigenic components have been noted in local populations of the two species of fish.

An extensive comparative study of the distribution in fishes of antigens reacting with human agglutinins and anti-sheep serum has been made and will be reported in another paper.

It is possible that the fish cell agglutinins discovered in human serum may be of clinical interest, as for example in cases of allergies to fish foods.

The authors acknowledge financial support by the Scripps Institution of Oceanography and suggestions and encouragement from Dr. Carl L. Hubbs of that institution. Dr. K. S. Pilcher, Director of Research, Cutter Laboratories, Berkeley, California, kindly made available the human typing sera used in this work. The rabbit anti-tuna sera were prepared using tuna blood supplied through the efforts of Dr. John L. Kask and Fred C. June, U. S. Fish and Wildlife Service, Pacific Oceanic Fishery Investigations, Honolulu. The authors would like to thank Prof. R. D. Owen, California Institute of Technology, for suggestions concerning this manuscript.

Summary

1. Normal human sera were found to agglutinate the erythrocytes of the shiner sea-perch (*Cymatogaster aggregata*) and of the kingfish (*Genyonemus lineatus*).

2. Agglutination was shown to occur by virtue of four different antigens, each species having two of these four in its erythrocytes.

3. Of these, *Genyonemus* had an antigen with a specificity closely resembling the human B substance (designated here as F-1) and a species-specific antigen (F-2) peculiar to itself.

4. *Cymatogaster* had a species-specific antigen (F-3) and an antigen with a specificity resembling that of the Forssman antigen (F-4).

5. No individual variations were found in either fish with respect to these antigens.

LITERATURE CITED

Ball, E. G., 1933. Hemolytic action of silver occurring as an impurity in chemically pure sodium chloride. *Biol. Bull.*, **64**: 277–288.

Boyd, W. C., 1947. Fundamentals of immunology. Interscience Publishers Inc., New York.

Cotterman, C. W., 1944. Serological differences in the genus *Peromyscus* demonstrable with normal human sera. *Contributions from the Laboratory of Vertebrate Biol., Univ. of Michigan*, **29**: 1–13.

Cushing, John E., 1942. An effect of temperature upon antibody production in fish. *J. Immunol.*, **45**: 123–126.

Cushing, John E., 1945a. A comparative study of complement. I.The specific inactivation of the components. *J. Immunol.*, **50**: 61–74.

Cushing, John E., 1945b. A comparative study of complement. II. The interaction of components of different species. *J. Immunol.*, **50**: 75–89.

Cushing, John E., 1952a. The serological differentiation of fish bloods. *Science*, **115**: 404–405.

Cushing, John E., 1952b. Individual variation in the haemagglutinin content of yellowfin tuna and skipjack bloods. *J. Immunol.*, **68**: 543–547.

Gemeroy, Douglas G., 1943. On the relationship of some common fishes as determined by the precipitin reaction. *Zoologica*, **28**: 109–123.

Grubb, Rune, 1949. Some aspects of the complexity of the human ABO groups. *Acta Path. Microbiol. Scand., Suppl.*, **84**: 1–72.

IRWIN, M. R., 1947. Immunogenetics. *Advances in Genetics*, **1**: 133–159.

JONSSON, BENGT, 1944. Blutgrüppenstudien mit Japanischen und Schwedischen Aalseren. *Acta Path. Microbiol. Scand., Suppl.*, **54**: 456–464.

NUTTALL, G. H. F., 1904. Blood immunity and blood relationship. Cambridge Univ. Press, Cambridge.

RACE, R. R., AND RUTH SANGER, 1950. Blood groups in man. C. C. Thomas, Springfield, Ill.

SCHIFF, FRITZ, AND WILLIAM C. BOYD, 1942. Blood grouping technic. Interscience Publishers Inc. N. Y.

STORMONT, CLYDE, R. D. OWEN AND M. R. IRWIN, 1950. The B and C systems of bovine blood groups. *Genetics*, **35**: 134–161.

STRENG, OSV, 1938. Zur Kentniss der heterogenetischen und homologen Antigene. *Acta Path. Microbiol. Scand.*, **37**: 498.

TAMM, IGOR, 1952. Agglutination of fish and turtle erythrocytes by viruses. *Biol. Bull.*, **102**: 149–156.

TYLER, ALBERT, 1946. Natural heteroagglutinins in the body fluids and seminal fluids of various invertebrates. *Biol. Bull.*, **90**: 213–219.

TYLER, ALBERT, AND C. B. METZ, 1945. Natural heteroagglutinins in the serum of the spiny lobster, *Panulirus interruptus*. *J. Exp. Zool.*, **100**: 387–406.

WIENER, ALEXANDER S., 1943. Blood groups and transfusion. C. C. Thomas, Springfield, Illinois. Third edition.

WIENER, ALEXANDER S., 1951. Origin of naturally occurring haemagglutinins and hemolysins: A review. *J. Immunol.*, **66**: 287–295.

PHOTOREACTIVATION IN COLPIDIUM COLPODA[1]

A. C. GIESE, C. L. BRANDT, R. IVERSON AND P. H. WELLS[2]

Dept. of Biological Sciences, Stanford University, Stanford, California

Photoreactivation or reversal of the injurious effects of ultraviolet radiations (UV) by subsequent illumination with visible light has been observed in a variety of forms (for references, see Kelner, 1951 and Wells and Giese, 1950) but only one species of protozoan has been tested, namely *Paramecium aurelia* (Kimball and Gaither, 1951). Although considerable information has accumulated on photoreactivation and wave-length dependence of photoreactivating light, temperature dependence, photoreactivation time span etc. have been studied, the mechanism by which photoreversal occurs is still obscure. Additional data on forms other than those already studied may therefore be useful for this purpose. The present paper presents information of this kind on *Colpidium colpoda*, a ciliate protozoan. This form was chosen because it has a relatively high and regular division rate and is sufficiently large to be handled with ease and to be seen and counted with low powers of the dissecting microscope even with red light. Cultures were grown at 26° C. in 0.05% lettuce extract seeded the day before with *Pseudomonas ovalis*, in small tubes made from 0.4 mm. soft glass tubing, as described in detail elsewhere (Giese, 1945b).

Methods

The UV radiation from a quartz mercury arc run at atmospheric pressure and at 200 volts, 2.2 amperes, was passed through a natural quartz monochromator to resolve the light. The desired wave-length was focused manually on a slit between two razor blades. The light passing through the slit was reflected onto a horizontal quartz cell by a right angle quartz prism. Visible light of various wave-lengths was obtained by passing through a monochromator radiations from a GE medium pressure mercury arc. In each case the intensity of the light was measured by a thermopile calibrated against standard lamps (U. S. Bureau of Standards).

After irradiation with UV the colpidia were always handled in darkness or in dim red light from darkroom safety lights tested spectroscopically to make sure that only red light was transmitted, since some defective red bulbs transmit parts of the entire spectrum. Observations and counts were made under low power (× 6.6) with a B. and L. dissecting microscope. The number of individuals in a tube was counted three times daily and averaged for a given series. From these data the division rate was determined as described in previous reports (Giese, 1939). The light used for illuminating the colpidia while counting them was enclosed in a black cover and the beam was passed through a water cell and a Corning filter #2412 which transmits mainly wave-lengths 7400–6150 Å (cut off at λ5950 Å).

[1] Supported in part by funds made available by the Rockefeller Foundation.

[2] Now at the Department of Zoology, University of Missouri, Columbia, Missouri.

Experimental

The first experiments were designed to determine whether photoreactivation occurred. The colpidia were irradiated with a series of dosages of UV at λ2654 Å and then treated with blue light of λ4350 Å. It was apparent in the first experiments that photoreactivation of considerable degree had occurred. The nature of the experiments and the analysis of the data are illustrated in Figure 1. The dosages of λ2654 Å tried were 750, 1000, 1500 and 3000 ergs/mm². After the colpidia had been treated with a dosage of 3000 ergs/mm². at λ2654 Å, about 70% died and the remainder were greatly retarded in division. Yet in a sample of the same batch treated with a dosage of 113,000 ergs/mm². of blue light after the UV, all individuals survived and divided, the degree of retardation corresponding approximately to the amount found after somewhat less than 1000 ergs/mm². of the UV alone. Colpidia treated with UV plus visible in all cases act as though they had been given much

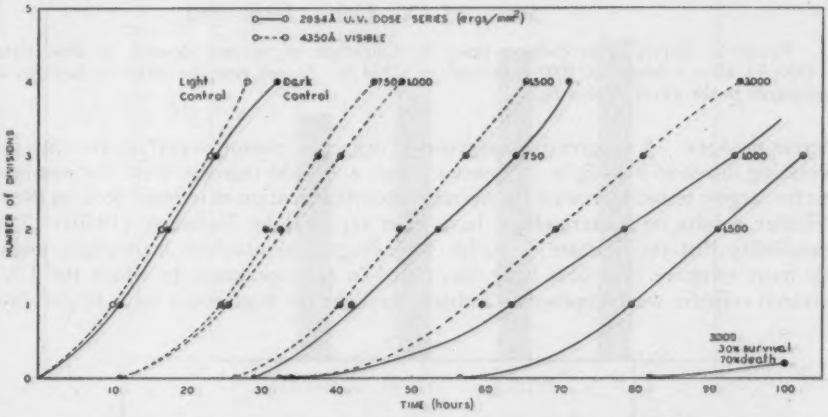


Figure 1. Effect upon division delay in Colpidium of various dosages of λ2654 Å with and without subsequent exposure to 56,900 ergs/mm². of blue light (4350 Å).

lower dosages of UV alone (see Kelner, 1949, dose reduction principle). Colpidia were exposed to a dosage of 1000 ergs/mm². of UV in all subsequent experiments because it measurably retards division, yet recovery of normal division rate occurs within a reasonable time (several days). When larger dosages are used the experiments last too long a time; it is then possible that various factors other than irradiation become limiting. Thus the medium containing bacteria deteriorates as a growth medium for Colpidium in 6–8 days. While transfer from old medium to new can be performed it involves undesirable handling of the animals.

The second series of experiments concerned itself with the amount of blue light necessary for maximal photoreversal of retarded division of Colpidium after UV treatment. In Figure 2 are given data for a series of experiments in which colpidia were irradiated with a dosage of 1000 ergs/mm². at λ2654 Å and were then illuminated with blue light (λ4350 Å) of approximately the same intensity but of dif-

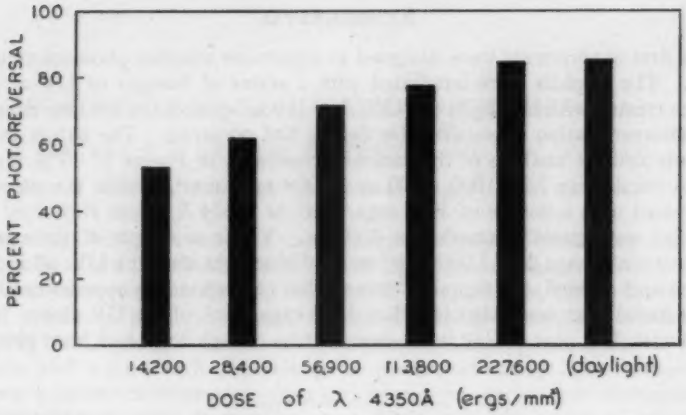


FIGURE 2. Effect upon division delay in Colpidium of various dosages of blue light (4350 Å) after a dosage of 1000 ergs/mm². of λ2654 Å. In one case the effect of daylight is compared to the effect of blue light.

ferent dosages. A progressively increasing degree of photoreversal occurs with increasing doses of blue light. However, even a 16-fold increase from the smallest to the largest tested increased the average photoreactivation only from 56% to 84%. Similar results on bacteriophage have been reported by Dulbecco (1950). The possibility that the mixture of visible wave-lengths as obtained in daylight might be more effective than blue light was tested in an experiment in which the UV-treated colpidia were exposed to indirect daylight for four hours on a bright day.

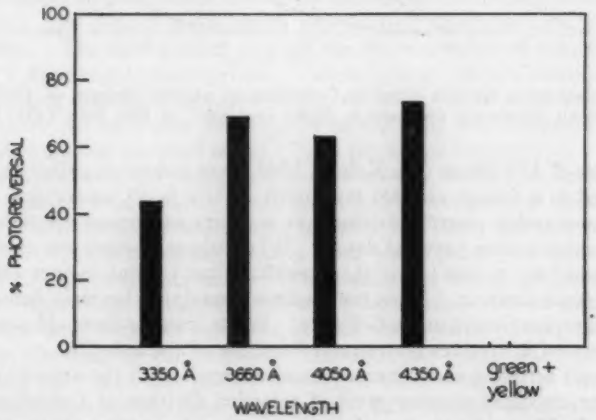


FIGURE 3. Effect upon division delay in Colpidium of a dosage of 56,900 ergs/mm². of various wave-lengths of long ultraviolet and visible light after a dosage of 1000 ergs/mm². of λ2654 Å.

The last bar in Figure 2 shows that reactivation after this large dosage of daylight is no greater than after 227, 600 ergs/mm². of blue light. While daylight might serve for photoreactivation studies, it is variable qualitatively and quantitatively; therefore a measured dosage of blue seemed preferable. While maximal photoreactivation with blue light might be desirable in some experiments, the time involved in giving a dosage of 227,600 ergs/mm². of blue light was too long to be practicable for most experiments; therefore a dosage of 56,900 ergs/mm². was settled upon as giving an adequate and satisfactory photoreversal, only 12% short of the maximal achievable.

To determine whether light other than blue might be more efficient for photoreversal in colpidia, the effect on photoreversal of several other wave-lengths (long ultraviolet: λ3350, 3660; violet, 4050; and yellow-green) available in the spectrum

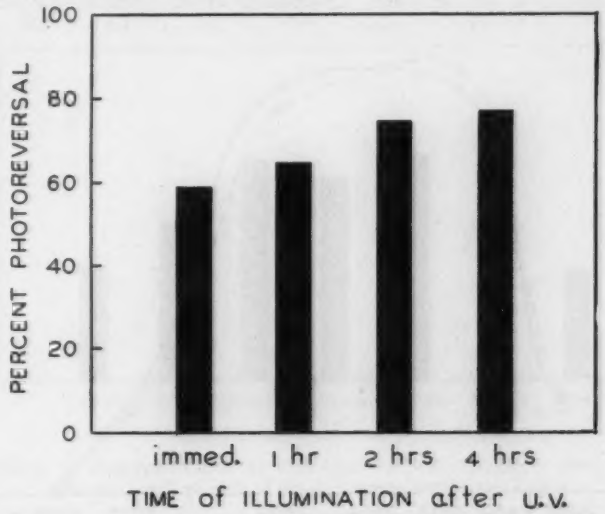


FIGURE 4. Effect of time lapse upon the degree of photoreactivation of division of Colpidium. The colpidia were given a dosage of 1000 ergs/mm². of λ2654 Å followed by a dose of 56,900 ergs/mm². of blue light (4350 Å) at the time specified.

of the mercury arc was compared with blue. The data are summarized in Figure 3. In all cases colpidia were first exposed to 1000 ergs/mm². of UV of λ2654 Å usually followed by 56,900 ergs/mm². of the photoreactivating light. It will be observed that the long UV (3350 and 3660 Å), the violet (4050 Å), and the blue (4350 Å) are quite effective for photoreversal. On the other hand yellow-green has no effect or possibly a slightly injurious effect. Since blue occurs in high intensity in the mercury arc spectrum, it was used for reactivation in all succeeding experiments except in a few performed with the whole visible spectrum.

In all cases a lapse of time occurred between the time of irradiation and the time of photoreversal. Since in studies on bacteria it was found by Kelner (1949) and others (*e.g.*, Novick and Szilard, 1949) that the time during which photoreversal

can be achieved is relatively short at room temperature and in active cultures, the possibility arose that in the experiments reported here, the time between irradiation with UV and photoreactivation was too long. To determine whether any such loss in the capacity for photoreactivation occurred within a few hours following UV irradiation, illumination was performed at various times up to four hours after UV treatment. The amount of photoreactivation was observed to be even greater in some cases than if the blue light had been applied immediately after UV irradiation of colpidia [3] (Fig. 4). For the present series of experiments, it was considered quite adequate to perform the exposure to visible light within several hours of the ir-

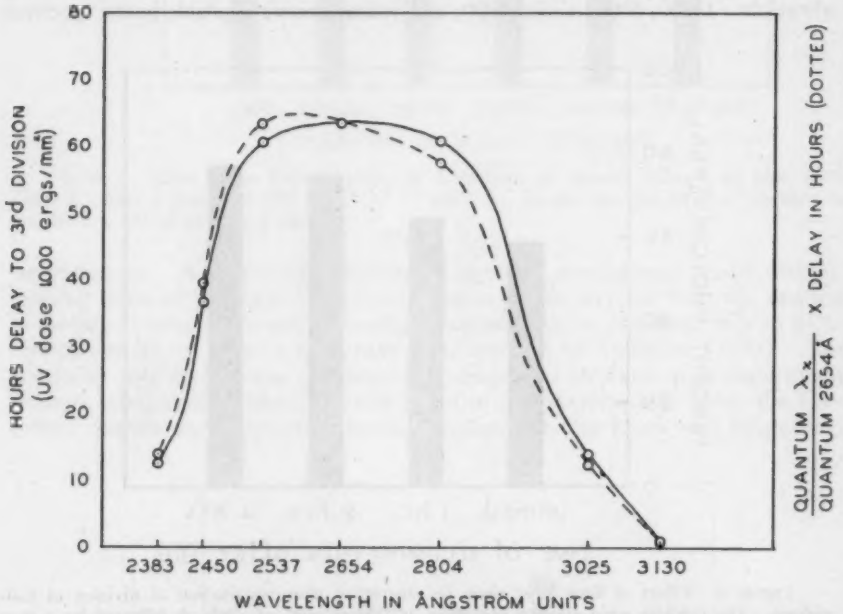


FIGURE 5. Relative efficiency of various wave-lengths of ultraviolet light upon division of Colpidium. The colpidia were irradiated with a dose of 1000 ergs/mm². at each of the wave-lengths. Comparison of relative efficiency on the basis of quanta is shown in the dotted curve.

radiation with UV. Because of the possibility of an increase in photoreactivation with lapse of time after UV exposure, it was necessary to illuminate with blue light at about the same time lapse after irradiation in order that the experiments might be comparable.

The final set of experiments was planned as a comparative study of the degree of photoreactivation following exposure at various UV wave-lengths. In most cases a dosage of 56,900 ergs/mm². of blue light (4350 Å) was used for photoreac-

[3] Recent experiments carried out by C. L. Brandt (unpublished) indicate that the reactivation can be achieved for as long as 23 hours after UV treatment, probably until the first division after UV treatment but not thereafter.

tivation. Figure 5 shows the relative efficiency of a dosage of 1000 ergs/mm². of each of the various UV wave-lengths used in retarding the division of Colpidium. The effectiveness is compared in two ways: 1) on the basis of the energy incidence per/mm²., and 2) on the basis of the number of quanta per mm². The results give a crude action spectrum for the UV retardation of division. The most effective wave-lengths are 2537, 2654, and 2804 Å. Wave-lengths on the short end: 2450 and 2383 Å, and on the long end: 3025 and 3130 Å, are less effective in retarding division than are the intermediate wave-lengths. In Figure 6 is shown the degree of photoreactivation produced by exposing to a dosage of 56,900 ergs/mm². at λ4350 Å colpidia previously treated with 1000 ergs/mm². at each of the UV wave-lengths.[4] Illumination in all cases was performed shortly after irradiation.

Pretreatment of colpidia with blue light (dosage: 46,000 ergs/mm².) does not protect them significantly from subsequent exposure to UV (2654 Å, 1000

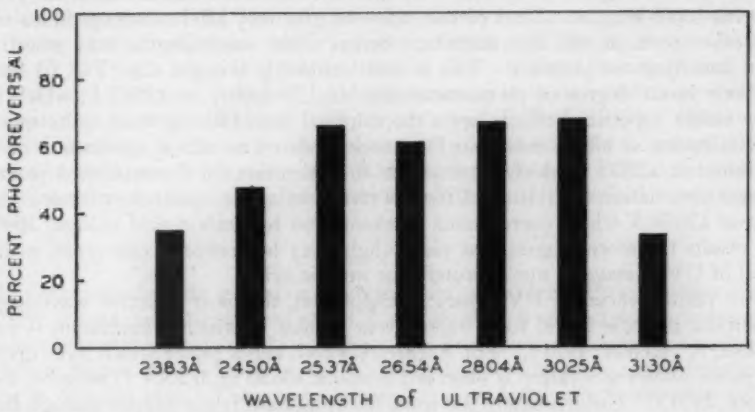


FIGURE 6. Degree of photoreactivation by blue light (56,900 ergs/mm².) after a dosage of 1000 ergs/mm². at each of the UV wave-lengths specified.

ergs/mm².). Colpidia so treated showed almost the same degree of retardation as those exposed to UV alone. Pretreatment with four hours of daylight before exposure to UV, however, produced some reduced sensitivity to UV applied thereafter. This phenomenon has been called *photodesensitization* by Weatherwax and Dobson (personal communication, 1951) who first observed it in *Escherichia coli* strain B. The decrease in sensitivity in Colpidium was only a small fraction of the photoreversal achieved by exposure to visible light after UV treatment.

DISCUSSION

UV affects division of Colpidium in two ways: a lag appears before division occurs, and, subsequent to larger doses, the rate of division is reduced. Treatment with visible light (or long UV) to a considerable extent reverses both of these ef-

[4] Colpidia treated with radiations of λ3025 Å were illuminated with only 42,000 ergs/mm². of blue light owing to an error in calibration, discovered on checking the calculation. This may introduce an error of several per cent in the value for this wave-length in Figure 6.

fects. UV treatment also caused a lag before division starts and a decrease in division rate in *Paramecium caudatum* (Giese, 1939) and in *Blepharisma undulans* (Giese and Reed, unpublished and Hirshfield and Giese, unpublished). In some ciliates, however, UV treatment also induced a cessation of division which occurs after one, sometimes after two, and, rarely, after three divisions have occurred subsequent to UV treatment. This was seen in *P. aurelia* and in *P. multimicronucleatum* (Giese and Reed, 1940; Kimball and Gaither, 1951). A similar effect has recently been found in *Tetrahymena geleii* (E. Christensen, unpublished), but it was never observed in Colpidium. The reason for this difference in effects of UV on different species of protozoa is not clear.

The different degrees of photoreactivation by blue light after irradiation of colpidia with various wave-lengths of the UV indicate that various UV wave-lengths may produce qualitatively different types of effects. This in turn may possibly be interpreted as being due to alteration of different chemical constituents of the cell by different wave-lengths. Thus certain wave-lengths may affect nucleoproteins more than other protoplasmic constituents, whereas other wave-lengths may selectively affect unconjugated proteins. This is most strikingly brought out (Fig. 6) by the relatively lesser degree of photoreactivation of UV injury at λ2383 Å, which produces rather superficial effects upon the colpidia, immobilizing them quite quickly. Immobilization of ciliary action in Paramecium shows an action spectrum with one maximum at λ2800 Å which corresponds to absorption by unconjugated proteins, whereas retardation of division of the cell shows an action spectrum with one maximum at λ2654 Å which corresponds to absorption by nucleic acid (Giese, 1945a). The results therefore suggest that visible light may be primarily concerned with reversal of UV damage to nucleoproteins or nucleic acid.

For photoreversal of UV injury in Colpidium, the most effective wave-lengths are similar to those found for *Streptomyces griseus* in which a maximum is found at λ4360 Å (Kelner, 1951). For *Escherichia coli*, and a phage which lives upon it, maximum photoreactivation is observed at about λ3650 to 3750 Å (Dulbecco, 1950; Kelner, 1951). Unfortunately the work on Colpidium is not precise enough to enable one to determine a detailed photoreactivation action spectrum. The main uncontrollable variable is the change in physiological state of the colpidia during the growth cycle. If one could obtain animals in exactly the same physiological state, this could be avoided. However, it is difficult to get experimental animals in exactly the same phase in the growth cycle since after a few divisions, the progeny of a single Colpidium begin to divide at a rhythm quite different from one another. The physiological state may have a profound effect on sensitivity to UV. Thus retardation of the third division of a vigorous group of colpidia (*e.g.*, those in which the three divisions occur within 20 to 22 hours in the control) by exposure to a dosage of 1000 ergs/mm². at λ2654 Å may be only 20 hours, whereas less vigorous colpidia (*e.g.*, those in which the three divisions occur in the control within 24 to 26 hours) given a similar dosage of UV may show a retardation of the third division of about two to three times the first group. Fortunately for experiments on photoreactivation, the dose reduction is similar; that is, the relative degree of recovery induced by visible light is similar regardless of the UV sensitivity of the colpidia. In recent experiments in which the colpidia were systematically starved before exposure to UV, great increases in sensitivity to UV have been observed with starvation

(Giese, Jacobson and Shepard, unpublished). The nature of the cellular change which causes such a striking difference in sensitivity has not been determined.

Since very large dosages of visible light are necessary for photoreversal of UV injury in Colpidium, it would appear that the substance or bond absorbing the visible light and bringing about the photoreversal is present in very small quantities or that the reaction is very inefficient because of the small amount of energy available in quanta of visible light. Since photodesensitization is so slight in Colpidium, it would appear likely that the compound concerned with photoreactivation appears in the form in which it can fruitfully absorb visible light only after absorption of UV. Shugar's studies (1951) suggest that one cellular compound absorbing light in the region of the spectrum effective in photoreactivation may be the enzyme D-glyceraldehyde-3-phosphate dehydrogenase, present with its reduced coenzyme. Absorption of the light by the adsorbed coenzyme results in reduction of the enzyme; light is ineffective if chemical reduction of the enzyme by cysteine is first performed.

Good photoreversal of UV injury in Colpidium by treatment with blue light even four hours after irradiation with UV light indicates that the injurious substance formed by UV light has a long life and that it does not exert its effect until some time after its production. At first sight this seems to be quite contrary to what has been found in bacteria (Kelner, 1949; Novick and Szilard, 1949). However, a normal Colpidium divides only about once every seven to eight hours at 26° C., whereas bacteria may divide every 30 to 60 minutes. When the inter-divisional time is large, the time during which photoreaction of UV injury can be achieved may also be long.

Summary

1. *Colpidium colpoda*, grown on a single strain of bacteria (*Pseudomonas ovalis*) in 0.05% lettuce medium buffered at pH 7, was irradiated with various wave-lengths of monochromatic ultraviolet (UV) light. UV treatment produces a characteristic lag before beginning of fission and, following larger dosage, a characteristic decrease in division rate. The greatest retardation of division was observed following exposure to λ2537, 2654 and 2804 Å.

2. The retarding effects of UV light at each wave-length were progressively greater with increasing dosages until division of treated colpidia was prevented. A dosage of 3000 ergs/mm². at λ2654 Å prevented division and resulted in death of 70 per cent of the colpidia.

3. Illumination with visible light of λ4350 Å effectively reversed UV injury resulting from a dosage of 1000 ergs/mm². at each of a number of wave-lengths of UV. For photoreactivation a dosage of 56,900 ergs/mm². of blue light (4350 Å) was used.

4. The degree of photoreversal by blue light of injury produced by wave-lengths 2537, 2654, 2804 and 3025 Å was larger than for wave-lengths 2383, 2450 and 3130 Å.

5. Pretreatment with blue light before exposure to UV light did not protect the colpidia; in other words a lag in, or retardation of, division comparable to that found for animals given the UV treatment alone was observed. However pretreatment with bright daylight protected colpidia to some extent from subsequent UV exposure.

6. Wave-lengths 3350, 3660 (long UV), 4050 (violet), and 4350 Å (blue) were effective in photoreversal of UV-injury in colpidia, but the yellow and green portions of the mercury arc spectrum were ineffective.

7. A large degree of photoreversal of UV-injury in colpidia could be induced even four hours after UV treatment, indicating persistence of the UV effect.

LITERATURE CITED

DULBECCO, R., 1950. Experiments on photoreactivation of bacteriophage inactivated by ultraviolet radiations. *J. Bact.*, **59**: 329–347.

GIESE, A. C., 1939. Ultraviolet radiations and cell division. The effects of λ2654 and 2804 Å. *J. Cell. Comp. Physiol.*, **13**: 139–150.

GIESE, A. C., 1945a. The ultraviolet action spectrum for retardation of Paramecium. *J. Cell. Comp. Physiol.*, **26**: 47–55.

GIESE, A. C., 1945b. A simple method for division rate determinations in Paramecium. *Physiol. Zool.*, **18**: 155–161.

GIESE, A. C., AND E. A. REED, 1940. Ultraviolet radiations and cell division. Variations in resistance to radiations with stock, species and nutritional differences in Paramecium. *J. Cell. Comp. Physiol.*, **15**: 395–408.

KELNER, A., 1949. Experiments on visible light on the recovery of *Streptomyces griseus* conidia from ultraviolet irradiation injury. *Proc. Nat. Acad. Sci.*, **35**: 73–79.

KELNER, A., 1951. Action spectra for photoreactivation of ultraviolet irradiated *Escherichia coli* and *Streptomyces griseus*. *J. Gen. Physiol.*, **34**: 835–852.

KIMBALL, R. F., AND N. GAITHER, 1951. The influence of light upon the action of ultraviolet radiations on *Paramecium aurelia*. *J. Cell. Comp. Physiol.*, **37**: 211–231.

NOVICK, A., AND L. SZILARD, 1949. Experiments on light reactivation of ultraviolet inactivated bacteria. *Proc. Nat. Acad. Sci.*, **35**: 591–600.

SHUGAR, D., 1951. Photoreactivation in the near ultra-violet of D-glyceraldehyde-3-phosphate dehydrogenase. *Experimentia*, **7**: 26–28.

WELLS, P. H., AND A. C. GIESE, 1950. Photoreactivation of ultraviolet light injury in gametes of the sea urchin, *Strongylocentrotus purpuratus*. *Biol. Bull.*, **99**: 163–172.

THE SIGNIFICANCE OF THE DARK PERIOD IN THE PHOTOPERIODIC RESPONSE OF MALE JUNCOS AND WHITE-THROATED SPARROWS[1]

CHARLES E. JENNER AND WILLIAM L. ENGELS

Department of Zoology, University of North Carolina, Chapel Hill, North Carolina

With a very few notable exceptions, all published studies on animal photoperiodism assign the critical role, either explicitly or implicitly, to the length of the daily period of light, acknowledging only the contributory roles of such attributes of light as wave length and intensity, and testing only such possibly modifying factors as temperature, food and activity. This conventional point of view quite disregards the existence of an accompanying, inverse variable, namely, the length of the daily period of darkness.

It is now well established that the length of the period of darkness is critically important in the photoperiodic responses of plants. So-called "long-day" plants, which flower on a relatively long daily period of light, do not *require* the long light period for flowering, since they will flower on a "short-day" if the long period of darkness defining the short day is interrupted by even a brief period of light (*e.g.*, 50 foot-candle minutes; Borthwick *et al.*, 1948).

Of the few studies on animals which incorporate data pertinent to this point, the earliest is that of Shull (1929) on aphids. He found that on a 12-hour day (12-hour night) almost all of the progeny produced by his aphids were winged, whereas on a 14-hour day (10-hour night) practically all the offspring were wingless. But he obtained the same sharply differentiated responses by exposing the aphids to 6-hour periods of light alternating either with 12-hour or 10-hour periods of darkness. Since the length of the light period was identical in the latter two cases, the difference in response must be ascribed to the difference in the length of the dark periods. In Dickson's (1949) extensive study of the photoperiodic control of diapause in the oriental fruit moth, he concluded that although the length of the light and the dark periods are both important factors, in nature diapause is caused mainly by the number of hours of darkness passing a critical minimum point. One of us (Jenner, 1951) has shown that on a short day certain snails fail to lay eggs, or lay very sparingly, but if on a shorter day the dark period is interrupted with light (total light per 24 hours equal to or less than the short-day controls), eggs are laid as abundantly as when the snails are on a long day. He has since obtained comparable results with the fresh-water shrimp, *Palaemonetes*. Hammond (1951) found that on a schedule of 7 light-hours alternating regularly with 5 dark-hours female ferrets come into oestrus ahead of ferrets exposed to normally increasing winter daylengths; this schedule might possibly be interpreted as a combination of short days (7 hours) with short nights (5 hours), but obviously there may be other very different interpretations. Hart (1951), also using female ferrets, found that by adding

[1] An abstract of this paper appeared in the Anatomical Record, volume 113 (1952).

just one hour of light at midnight to the normally increasing day-lengths of winter the oestrus cycle was greatly accelerated. However, due to lack of an adequate control group, it cannot be determined whether his results are to be ascribed to the extra hour of light or to the interruption of the dark period.

To the best of our knowledge, there are no other published data bearing on this problem in animals, either invertebrate or vertebrate, and it is apparent that, for vertebrates at least, the evidence is not only meager but at most inconclusive.[2] The experiments to be described below were designed expressly, then, to answer the question: Is it the length of the daily period of light alone which is critical, as has been generally supposed, or does the length of the daily period of darkness play a definitive role in the photo periodic mechanism of vertebrates?

Materials and Methods

Slate-colored juncos (*Junco hyemalis*) and white-throated sparrows (*Zonotrichia albicollis*) were trapped during the winter of 1951–52 in the Arboretum on the university campus and on the grounds of the zoology building, and were distributed in six cages, six to eight birds in each cage. A 40-watt, 48-inch daylight fluorescent tube was suspended about one foot above each cage; these lights were turned on and off by automatic 24-hour clock timers. The cages were arranged in pairs, back-to-back; one cage of each pair contained juncos, the other held sparrows.

Each pair of cages was in a separate room, two of which were light-tight; in the third room the lights went on before dawn and remained on until after dark, for a total of 16 hours. The birds in this latter room are referred to as Group A (long day—short night) or long-day controls. In one of the light-tight rooms, the lights were on continuously for 10 hours in each 24; during the other 14 hours the birds were in complete and continuous darkness. These birds are referred to as Group B (short day—long night) or short-day controls. In the second light-tight room, the lights were operated by two timers in such a way that the dark period following 8¼ hours of continuous light was interrupted by a second light period of 1¾ hours. Thus, although the total hours of light per 24 were the same as in Group B, namely 10 hours, the periods of continuous darkness were only 7 hours rather than 14 hours as in B. Birds from this room are referred to as Group C (short day—interrupted night). These three light schedules are shown diagrammatically in Figure 1. The lights delivered 25–35 f.c. on the floor and perches. Group A birds were exposed occasionally to higher and more variable intensities.

The cages were constructed entirely of ¼-inch hardware cloth, and measured 2 ft. × 2 ft. × 3 ft. Each was provided with two perches made of ¼-inch dowelling, 2 feet long and placed about 5 inches above the floor of the cage, which rested on small wooden blocks.

Fine-cracked corn was present at all times in the food trays, which also contained a commercial "bird gravel." Pablum was supplied daily in a separate dish; both

[2] While this paper was in press, Kirkpatrick and Leopold (Science, volume 116, pp. 280–281) reported the induction of full sexual activity in quail held on short day—interrupted night light schedules essentially similar to those employed by us, and concluded, as we do, that "the duration of the dark period is a major controlling factor of photoperiodic responses."

species seemed to prefer this to the grain. During most of the experimental period peanut hearts were mixed with the grain, as well as small amounts of a commercial "bird seed." A continuous supply of water was assured by the use of quart jars inverted on baby-chick watering pans. The birds appeared to remain healthy under these conditions; all that were sacrificed in the course of the experiment were at least somewhat fat. Apart from a few juncos that died within 12 hours of capture, only four birds died in the cages out of a total of 38 caged birds.

Maximum and minimum temperatures were recorded daily in each room, from thermometers placed next to the floor of each pair of cages. The temperature record (° F.) is as follows:

	Average maximum	Average minimum
A	74.5 (67–81)	66.5 (60–72)
B	75.2 (72–79)	71.6 (69–76)
C	73.5 (71–77)	70.0 (67–74)

The slight differences, especially as between the control room B and experimental room C, are not regarded as significant (cf. Burger, 1948).

Groups A and B were in our personal laboratories, group C in a small, otherwise unoccupied room. To avoid disturbance to the birds the A and B cages were partly shrouded with cloth draped from the frame supporting the lights, and this effectively shielded the birds from sight of movements in the room. They did not appear to notice the sound of conversation or other noises; the birds of group A, especially, frequently sang throughout the day. Since the results place group B in sharp contrast with both A and C, the isolated and relatively undisturbed position of the C cages is not regarded as relevant.

Juncos were trapped on January 4, 5 and 6, 1952, banded and placed under treatment on the dates of capture. All the juncos were killed on March 1, after 55–57 days of treatment. The initial group of white-throated sparrows was trapped during the period December 17–20, 1951, and the experiment begun on December 19. Additional sparrows were trapped and added to the cages between December 30 and January 3. These latter birds were banded to distinguish them from the original, unbanded lot, but the loss of some bands in the C cage made uncertain the length of treatment for the first C birds killed, on January 17. The sparrow experiment was terminated on February 21. Birds were classed as adults or immatures on the basis of the frontal bones (Miller, 1946) at the time of sacrifice. It is possible that some which were "immatures," *i.e.*, birds of the year, at the start of the experiment had become "adults" on this criterion by the end of the experimental period.

The birds were killed with chloroform, in groups of three to six. Both testes of each male were removed with fine forceps, placed together in a tared weighing bottle, weighed on a balance to the nearest 0.1 mg. and then immediately flooded with Bouin's fluid. Long and short diameters of each testis were subsequently measured for determination of volumes, and one (usually the larger) of each pair was sectioned at 10 μ; the sections were stained with Ehrlich's hematoxylin, some counterstained with eosin. The volumetric data in Tables I and III refer to the sectioned testis, weights to the pairs of fresh testes. Maximum tubule diameters were measured from the sections with an ocular micrometer, and the progress of spermatogenesis recorded according to the stages of Blanchard (Blanchard, 1941;

TABLE I

Some data on results of artificially imposed lengths of day and night on male slate-colored juncos (Junco hyemalis)

Date collected (1952)	Duration of treatment (days)	Weight both testes (mg.)	Volume sectioned testis ($mm.^3$)	Max. tubule diam. (μ)	Stage of spermatogenesis
Field Controls					
Jan. 6	—	1.6	0.64	55	I
Jan. 8 (immature)	—	0.6	0.31	52	I
Jan. 11 (immature)	—	0.4	0.19	42	I
Feb. 28	—	4.7	2.05	100	IV
Feb. 28	—	1.4	0.47	75	II
Mar. 5	—	1.2	0.68	65	II
Mar. 6	—	1.9	1.06	82	III
Mar. 6	—	2.3	0.82	78	III
Mar. 6	—	1.8	0.74	75	III
Mar. 6	—	1.4	0.74	88	III
Mar. 6	—	0.6	0.58	82	III
Mar. 6	—	0.8	0.43	75	III
Group A. "Long day—short night"					
Jan. 4	57	348.8	205.0	575	VII
Jan. 4	57	188.5	93.0	525	VII
Jan. 5	56	245.2	139.0	600	VII
Jan. 5	56	160.1	94.0	500	VII
Jan. 6	55	184.8	107.0	450	VII
Group B. "Short day—long night"					
Jan. 4	57	0.9	0.52	78	III
Jan. 4	57	0.3	0.23	58	II
Jan. 5	56	1.6	0.76	91	III
Jan. 5	56	0.7	0.38	72	II
Jan. 6	55	0.8	0.42	65	II
Group C. "Short day—interrupted night"					
Jan. 4	57	128.5	70.5	400	VII
Jan. 4	57	76.2	37.5	350	VI
Jan. 4	57	50.8	25.5	300	VI
Jan. 5	56	77.7	42.5	375	VI
Jan. 5	56	4.2	1.84	125	IV
Jan. 6	55	18.2	8.10	200	V

Blanchard and Erickson, 1949). These stages may be characterized, briefly, as follows:

I. Inactive; interstitial lipoid Leydig cells absent, or, if present, small, not recognizable in ordinary preparations.

II. First appearance of recognizable Leydig cells.

III. Marked increase in number of spermatogonia; Leydig cells numerous.
IV. A few primary spermatocytes in synapsis in each tubule cross-section.
V. Predominance of primary spermatocytes in synapsis.
VI. (early). Predominance of spermatids.
VI. (late). Spermatids in transformation, much cellular debris in lumen of tubule, many sperm bundles but fewer sperm per bundle than in fully mature condition.
VII. Breeding condition; tubules packed with sperm bundles; mature sperm free in lumen.

Results

Slate-colored juncos (*Junco hyemalis*). (Fig. 2; Tables I and II). The field controls demonstrate that at the beginning of the experiment the testes were minute and spermatogenically inactive.

Group A. After 8 weeks on a "long day—short night" schedule, spermatogenesis was complete in each of the five males in this group. The tubules were swollen, each with a large lumen, and packed with bundles of mature sperm.

Group B. Recrudescence of the testis was initiated in each of the five males on a "short day—long night" schedule after 8 weeks. However, none had developed beyond the stage of marked increase of spermatogonia (stage III) and only two had even reached that stage. The testes were still minute, but the tubules had enlarged slightly.

Group C. After 8 weeks on the experimental "short day—interrupted night" schedule, testes of four of the six males contained fully developed sperm in numerous bundles. One of these four corresponded to the long-day group A testes, with spermatogenesis complete; in the other three many spermatids were still in the process

Table II

Stages of spermatogenesis attained by slate-colored juncos, artificially lighted from Jan. 4–6 to Mar. 1. Each "x" indicates one bird

Stages	Field Jan. 6–11	A Long day	B Short day	C Interrupted night	Field Feb. 28–Mar. 6
VII		xxxxx		x	
VI late				xxx	
VI early					
V				x	
IV				x	x
III			xx		xxxxxx
II			xxx		xx
I	xxx				

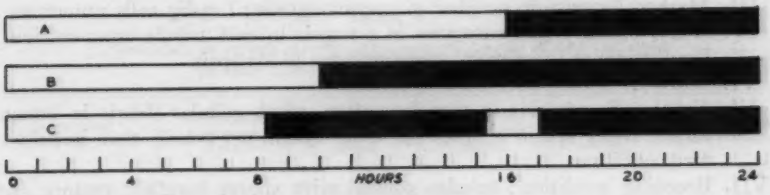


FIGURE 1. Schematic diagram of light schedules per 24 hours. A = long-day control; B = short-day control; C = "interrupted night." Total number of light hours in C the same as in B, but through interruption of darkness in C, periods of continuous darkness are one hour less than in A.

of metamorphosis, although there were many small bundles of metamorphosed sperm in each tubule section. In the testes of the remaining two experimental birds the first maturation division was under way.

Most of the juncos field-trapped and sacrificed at about the termination of the experiment were in spermatogenic states comparable to those attained by our short-day controls, group B. Only one of nine had reached the stage of initiation of spermatocyte division. That one is thus similar to the least developed of our experimental birds of group C. At this time, day lengths were approaching 12 hours (sunrise-sunset), and these outside birds had been exposed, for almost four weeks, to day lengths exceeding the 10 hours allowed our experimental and short-day control birds.

White-throated sparrows (*Zonotrichia albicollis*). (Tables III and IV.) The results, at first glance, may not appear to be as convincing as in the case of the juncos.

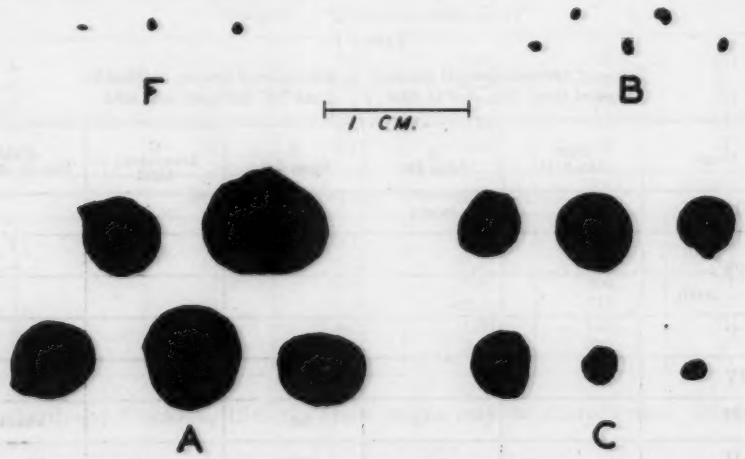


FIGURE 2. Relative sizes of junco testes in early January (F = field controls) at start of experiment, and after 55–57 days on the experimental light schedules of Figure 1; each testis is from a different bird. From a negative photographic print. A = long-day control; B = short-day control; C = "interrupted night."

Most of the birds were sacrificed at the end of four weeks, a date which proved to be perhaps too early, and left very few birds to be treated for seven weeks. Then, due to the loss of some bands, the group C birds sacrificed at the end of four weeks included a few undetermined individuals that had been in the cages only 15–18 days. Nevertheless, we believe, especially in the light of the results with juncos, that these

TABLE III

Some data on results of artificially imposed lengths of day and night on male white-throated sparrows (Zonotrichia albicollis)

Date collected (1951–52)	Duration of treatment (days)	Weight both testes (mg.)	Volume sectioned testis ($mm.^3$)	Max. tubule diam. (μ)	Stage of spermatogenesis
Field Controls					
Dec. 29	—	1.9	1.01	58	I
Dec. 29	—	2.1	0.98	55	I
Dec. 29	—	2.2	0.89	55	I
Dec. 29 (immature)	—	0.5	0.42	46	I
Feb. 28	— *	4.0	3.04	68	II
Feb. 29	—	5.5	2.35	65	II
Feb. 29	—	0.6	0.55	49	I
Group A. "Long day—short night"					
Dec. 17–20	29–30	161.0	87.0	420	VII
Dec. 17–20	29–30	100.9	47.9	330	VI
Dec. 17–20	29–30	81.0	31.4	390	VI
Dec. 30	52	274.1	200.0	650	VII
Jan. 1	51	151.5	67.5	520	VII
Jan. 3	49	258.7	143.5	600	VII
Group B. "Short day—long night"					
Dec. 17–20	29–30	2.7	1.20	68	II
Dec. 17–20	29–30	2.7	1.06	65	I
Dec. 17–20	29–30	1.9	1.06	71	II
Dec. 17–20	29–30	1.9	0.95	62	I
Dec. 17–20 (immature)	29–30	—	1.08	65	I
Jan. 1	51	0.5	0.34	58	II
Jan. 3	11*	1.9	0.95	62	II
Group C. "Short day—interrupted night"					
Dec. 17–20	29–30	5.6	3.32	81	III
Dec. 17–20	29–30	3.8	1.20	62	II
Dec. 17–20 (immature)	29–30?	2.3	0.95	81	III
Dec. 17–20 (immature)	29–30	1.9	0.78	71	II
Dec. 17–20 (immature)	29–30	1.2	0.84	68	II
Jan. 3	49	52.1	31.2	290	VI
Jan. 3	49	9.4	10.3	160	IV

* = died in cage; ? = some of these collected Dec. 20–Jan. 3, treated only 15–18 days (bands lost).

data for white-throated sparrows do show that the interruption of the long dark period resulted in an acceleration of the spermatogenic process. At the beginning of the experiment (field controls), the testes of all birds were minute and inactive.

Group A. On a "long day—short night" schedule, the testes of three birds had greatly increased in size after four weeks. In one of these the tubules were packed with sperm, as in the breeding condition; in the other two the tubules were greatly enlarged, with many spermatids and a few sperm. After 7 weeks, the testes of three additional males had reached full breeding condition.

Group B. On the "short day—long night" regime, after four weeks, the testes of the three most advanced males showed just the beginning of recrudescence; in three others, the testes still were inactive. Even after 7 weeks, the single male left in this group had not advanced beyond the earliest stage of recrudescence (first appearance of interstitial Leydig cells).

Group C. Of the experimental males on a "short day—interrupted night," after four weeks the testes of one were definitely enlarged, and spermatogenesis had reached the stage of marked increase of spermatogonia. In another male the testes were still small, but the tubules showed a definite although slight increase in diameter and spermatogenesis had likewise reached the multiplication stage (III). The other three males had begun recrudescence. No testis was still in the inactive state, despite the fact that some of these birds (individuals unknown) had been treated for only 15–18 days. Two males were treated for 7 weeks; in both of these the testes had increased very considerably in size. One had reached the penultimate stage of spermatogenesis, with swollen tubules packed with spermatids; in the other some spermatocytes were beginning to divide. This latter condition is the one

TABLE IV

Stages of spermatogenesis attained by white-throated sparrows under artificial lighting

Stages	Field Dec. 29	A Long day	B Short day	C Interrupted night	Field Feb. 28–29
VII		a bbb			
VI late					
VI early		aa		b	
V					
IV				b	
III				aa	
II			aaa b	aaa	xx
I	xxxx		aaa		x

"a", "b" and "x" each denote one individual; "a" = experimental period of 29–30 days (Dec. 19–20 to Jan. 17), and "b" = experimental period of 49–52 days (Dec. 30–Jan. 3 to Feb. 21).

which, in a related species, either immediately precedes the beginning of spring migration, or, in non-migratory populations, accompanies pairing and onset of territorial behavior (*Z. leucophrys,* Blanchard, 1941; Blanchard and Erickson, 1949).

It is unfortunate that only one male was left in the control group B after seven weeks; but it is noteworthy that several days after the termination of the experiment three males collected in the field were far behind the less mature of the last two experimental birds. As explained above under juncos, the outside birds had been exposed to considerably more light per 24 hours than our groups B and C.

Discussion

Among our birds on the short day—interrupted night schedule, full testicular development was obtained not by exposing the birds to extra light but by breaking the dark period into two "short nights." It is therefore concluded that the length of the uninterrupted period of darkness is a controlling factor. Darkness evidently is not neutral or merely indifferent in the activation or inactivation of the photoperiodic response. Some sort of reaction must be going on in the dark which, probably, bears an inverse relationship to the reaction going on in light. If the dark period is long enough gonadotropic activity does not occur. If the dark period is short (or absent) gonadotropic activity does occur, but its occurrence is independent of the *length* of the light period, providing only that the duration of the light period is greater than some relatively low minimum. The significance of the length of the photoperiod is related primarily to the way in which it defines the length of the dark period. The species used in our experiments, and undoubtedly some other passerine birds as well, certainly do not require at least 11 to 12 hours daily light to complete spermatogenesis, as the conventional view would have it. What they do require, apparently, is that the daily dark period be less than 12 or 13 hours.

In our experiments the short day—interrupted night schedule obviously was not as effective in stimulating spermatogenic advance as was the long day—short night schedule, even though the dark periods (7 hrs.) were shorter than the dark period (8 hrs.) of the long day. Further investigation will be necessary to explain this difference. In our opinion the probable explanation is that the reaction which occurs in the light reaches a maximum at a quantity of light (intensity × time) greater than that employed for the dark-period interruption in our experiments. The fact that the testes of four of the six juncos on the interrupted-night schedule developed mature sperm (late stage VI or VII) in 8 weeks would seem to indicate that the interruption given (1¾ hours at about 30 f.c.) approached the light energy required for complete effectiveness. Therefore, if we consider that a light quantity slightly greater than 1¾ hours at 30 f.c. represents the order of magnitude of the light energy required to complete the light phase of the photoperiodic response, then it is obvious that in our experiments the light reaction reached the same maximum on the short day of 10 hours (group B) as on the long day of 16 hours (group A). The difference in response between these two day lengths must then be due to the difference in the length of the dark periods. We interpret this to indicate that whereas the light-phase of the reaction reaches a maximum rapidly, the dark-phase reaction takes place very slowly, and is therefore the time-measuring phase of photoperiodism.

There is an extensive literature on photoperiodism in birds and mammals. Conventionally it is thought that in these responses ". . . the light is received by the eye which sets off an unknown sequence of events terminating in stimulation of the pituitary" (Burger, 1949; p. 218). The pituitary in turn produces the gonadotropins which stimulate the gonads. The experiments reported here direct attention to what appears to be a characteristic feature of all photoperiodic responses, namely, that the duration of the dark period is critically important. The conventional view of animal photoperiodism must therefore be amended to include this concept.

The results of the present experiments, like those of Jenner (1951), demonstrate a similarity between plants and animals in their photoperiodic responses. The recognition of such a pattern of similarity emphasizes the desirability of understanding its basis.

Summary

1. Experiments were conducted with slate-colored juncos and white-throated sparrows to test the thesis that the dark period plays a definitive role in the photoperiodic response of these birds.

2. The results of the experiment using male juncos were as follows:

(a) Testes of birds on a short day (10 hours of light, 14 hours of darkness) underwent little or no development during the 8 weeks of the experiment.

(b) Exposure of other juncos to a long day (16 hours of light, 8 hours of darkness) during this same period resulted in their testes attaining full breeding condition.

(c) Experimental birds were placed on a light schedule which consisted of 8¼ hours of light followed by a dark period, the middle of which was interrupted by a second light period of 1¾ hours; thus they received a total (as in (a)) of 10 hours of light per 24-hour period, but dark periods were only 7 hours long. By the end of 8 weeks considerable testicular development had occurred among all males placed on this "interrupted-night" schedule. The testes of four birds had developed mature sperm (late stage VI or VII); the other two were somewhat less developed.

3. The response of white-throated sparrows placed under these same conditions was similar to that of the juncos.

4. The results show clearly that in the photoperiodic response of these birds there is a critically important dark-dependent phase. This dark-period dependence appears to be characteristic of all photoperiodic responses, in both plants and animals.

LITERATURE CITED

Blanchard, B. D., 1941. The white-crowned sparrows (*Zonotrichia leucophrys*) of the Pacific seaboard: environment and annual cycle. *Univ. Calif. Publ. Zool.*, **46**: 1–178.

Blanchard, B. D., and M. M. Erickson, 1949. The cycle in the Gambel sparrow. *Univ. Calif. Publ. Zool.*, **47**: 255–318.

Borthwick, H. A., S. B. Hendricks and M. W. Parker, 1948. Action spectrum for photoperiodic control of floral initiation of a long-day plant, Winter barley (*Hordeum vulgare*). *Bot. Gaz.*, **110**: 103–118.

Burger, J. W., 1948. The relation of external temperature to spermatogenesis in the male starling. *J. Exp. Zool.*, **109**: 259–266.

Burger, J. W., 1949. A review of experimental investigations on seasonal reproduction in birds. *Wilson Bull.*, **61**: 211–230.

DICKSON, R. C., 1949. Factors governing the induction of diapause in the oriental fruit moth. *Ann. Ent. Soc. Amer.*, **42**: 511–537.

HAMMOND, J., 1951. Control by light of reproduction in ferrets and mink. *Nature*, **167**: 150–151.

HART, D. S., 1951. Photoperiodicity in the female ferret. *J. Exp. Biol.*, **28**: 1–12.

JENNER, C. E., 1951. Photoperiodism in the fresh-water pulmonate snail, *Lymnaea palustris*. Thesis, Harvard University.

MILLER, A. H., 1946. A method of determining the age of live passerine birds. *Bird-banding*, **17**: 33–35.

SHULL, A. FRANKLIN, 1929. The effect of intensity and duration of light and of duration of darkness, partly modified by temperature, upon wing-production in aphids. *Arch. f. Entw.*, **115**: 825–851.

ON THE RELATION BETWEEN WATER TRANSPORT AND FOOD REQUIREMENTS IN SOME MARINE FILTER FEEDING INVERTEBRATES

C. BARKER JØRGENSEN [1]

Laboratory of Zoophysiology, University of Copenhagen, Copenhagen, Denmark, and Marine Biological Laboratory, Woods Hole, Mass.

Filter feeding invertebrates in the sea, *e.g.*, sponges, ascidians, most lamellibranchs, several gastropods and crustaceans, etc., obtain their food from the finely dispersed organic matter which is present in the water. A minor fraction of this organic matter is particulate and can be removed from the water by means of suitable filters, whereas a major fraction is "dissolved." It is not finally settled whether filter feeders obtain their food from the particulate fraction only, or whether they are also able to utilize the so-called dissolved organic matter in the sea. The view that filtering animals utilize the dissolved substances is mainly supported by calculations showing that particulate organic matter is not present in sufficient amounts to cover the energy requirements of the filter feeders. The calculations are based on measurements or estimates of rate of water transport, oxygen consumption and growth of the animals in question. The calculations are, however, somewhat uncertain because in no instance have water transport and metabolic rate been determined in the same specimen. It was therefore decided to measure filtration rate and oxygen consumption in the same individuals of some filter feeders and to repeat the calculations mentioned above.

Technique

The species used were the oyster, *Ostrea virginica*, and the ascidians *Ciona intestinalis* and *Molgula manhattensis*. Experiments were performed on one oyster at a time or on 5 Ciona or about 15 Molgula. The oysters were kindly supplied by Dr. Paul S. Galtsoff, Shellfish Laboratory, U. S. Fish and Wildlife Service, Woods Hole. They had been living in the tanks of the laboratory for about a month. Ciona and Molgula were used in the experiments after they had been adapted to the laboratory conditions for a day. Filtration rates were determined as previously described (Jørgensen, 1949a) by measuring the rates at which graphite particles ("Aquadag A") were removed by the animals. Graphite concentrations were measured by means of a Klett-Summerson photoelectric colorimeter. Control experiments showed that the particles used were retained almost completely in the filtering organs (Jørgensen and Goldberg, unpublished data). Oxygen was determined by the Winkler method.

[1] Lalor Fellow at the Marine Biological Laboratory during the summer of 1951.

Results

The results of the experiments are shown in Figures 1–4. The points in the graphs represent single determinations of filtration rate or oxygen consumption in liters of water or milliliters of oxygen per hour. It is seen that the rate of water transport was of the same order throughout the period of observation which lasted three to four days. In Ostrea and Molgula, the water transport was approximately

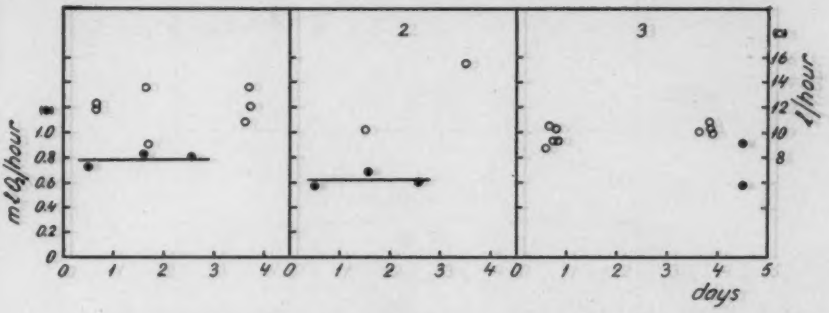


Figure 1. *Ostrea virginica.* ●Oxygen uptake in ml. per hour. ○Filtration rate in liters per hour. Experiments on three individuals.

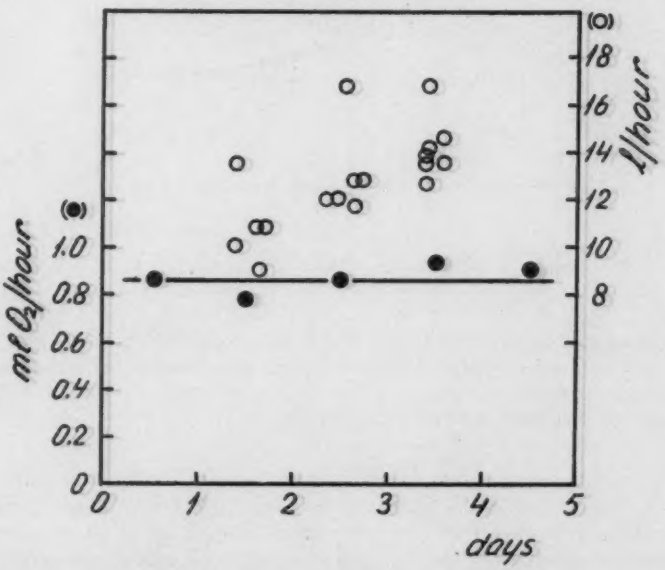
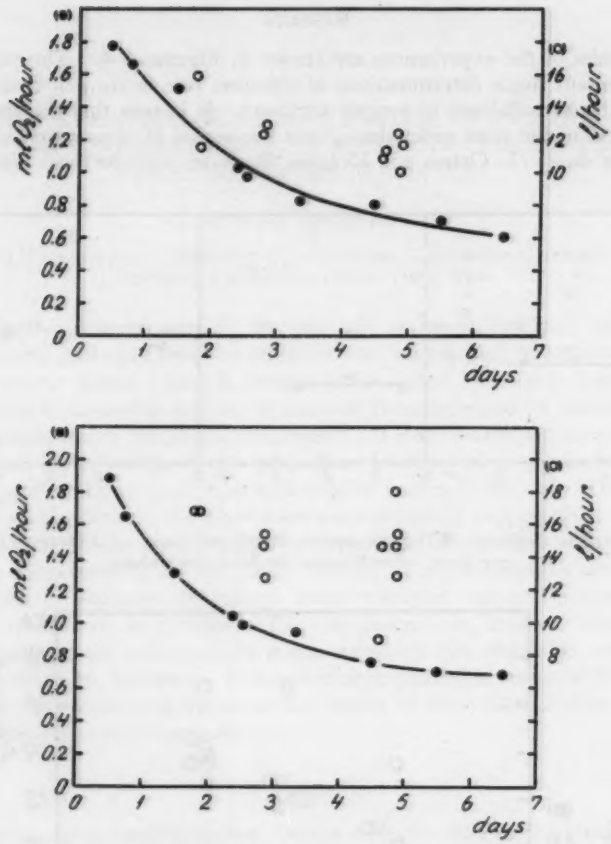


Figure 2. *Ciona intestinalis;* 5 specimens. ●Oxygen uptake in ml. per hour. ○Filtration rate in liters per hour.



FIGURES 3 AND 4. *Molgula manhattensis.* About 15 specimens. ● Oxygen uptake in ml. per hour. ○ Filtration rate in liters per hour.

constant from day to day. In Ciona, filtration rates were lower the first day than the two following days, probably because the animals became better adapted to the experimental conditions with time. The oxygen uptake was constant in the oyster and Ciona, but decreased with time in Molgula.

DISCUSSION

From Figures 1 and 2 it is seen that the oyster and Ciona pumped about 10 to 20 liters of water through their filtering organs for each ml. of oxygen uptake. In Molgula the ratio between water transport and metabolic rate is not well defined because oxygen was consumed at decreasing rates throughout the experiments. There is reason to believe that at least two factors are responsible for the decrease in

oxygen uptake. On arrival in the laboratory hydroids, bryozoans, algae, etc. growing on the mantle of the animals were removed. This procedure, which also involved separation of adhering animals, may have caused an increase in metabolic rate so that the rates of oxygen uptake have perhaps been abnormally high during the first days of the experiments. Mytilus when detached from their support by tearing of the byssus threads were also found to respond by strongly increasing their oxygen consumption. Thus, the steady decrease in metabolic rate of Molgula may just indicate a slow return towards values of oxygen uptake characteristic of undisturbed animals. However, poor food conditions may also be partly responsible for the continued decrease in oxygen consumption as pointed out below. Probably, therefore, normal metabolic rates are to be found somewhere in between the rates which were measured at the beginning and at the end of the experiments. If the measurements made on the third day of the experiments are used as "normal" values we find also in Molgula that about 10 to 20 liters of water are filtered for each ml. of oxygen uptake. Filtration rates of the same order were also measured in *Mytilus edulis*, *Pecten latiauritus* and *Ciona intestinalis* from coastal waters off Southern California (unpublished data).

The literature contains data on feeding rate and oxygen uptake in other filter feeders. Even when these data are not obtained from the same individuals and under identical experimental conditions, they are consistent with the results of the present study.

In the copepods *Calanus finmarchicus* and *Centropages hamatus*, feeding rate and oxygen uptake have been measured by Gauld (1951) and Raymont and Gauld (1951), respectively. From their figures, filtration rates of about 8 to 10 liters of water per ml. of oxygen uptake can be calculated.

Jørgensen (1949b) found that the silicious sponge *Halichondria panicea* filtered 65 ml. of water per hour and per mg. nitrogen (18–19° C.). Pütter (1914) measured the oxygen uptake in another silicious sponge, *Suberites massa*, to be 0.34 ml. per hour and per gram dry organic matter (22° C.). Assuming a content of 8 per cent nitrogen, this means 4.2 ml. O_2/hour/mg. nitrogen. Thus, if these values for water filtration and oxygen uptake are generally valid in silicious sponges, 15 liters of water are filtered for each ml. of oxygen uptake. The calcareous sponges *Grantia compressa* and *Sycon coronatum* showed pumping rates of about 140–200 ml./hour/mg. nitrogen (Jørgensen, 1949b). Probably, therefore, their filtration rate relative to metabolism is of the same order as that of the silicious sponges, perhaps even higher.

In phylogenetically unrelated filter feeders which use different filtering mechanisms, the rates of filtering and the metabolic rates have been adjusted to about the same ratio. Presumably the feeding rate, not the filtration rate as such, is primarily adjusted relative to the metabolic rate (and relative to the rate of growth). A constant ratio between rates of filtration and of oxygen uptake in different filter feeders therefore suggests that the concentration of available food in the sea where adaptation took place was about the same for all the filter feeders mentioned above. This conclusion of course rests on the assumption that the rate of filtration of the animals is the same in their natural environment as under the conditions of experiment. However, there is some reason to believe that this assumption holds. Food conditions must be better in the sea than in the running tap water of the laboratory

where much food is removed by other organisms growing in storage tanks and water pipes. The decrease in oxygen uptake observed in the course of an experiment in Molgula (Fig. 3 and 4) and in Mytilus and Pecten (unpublished) is probably partly due to starvation. However, filtration rates were not influenced by the reduction in food concentration. Filtration rates of lamellibranchs and ascidians are also independent of increased concentrations of food and other particles as long as the concentrations are not too high (Loosanoff and Engle, 1947, and others). Thus, if filtration rate can adjust to different food levels, this process must be slow. Hence, when measured with a suitable technique [2] the pumping rate and the oxygen uptake in undamaged animals which have been adapted to the laboratory conditions are therefore probably representative of the corresponding rates prevailing in the sea.

"Available food." The organic matter in the sea is present as organisms such as phytoplankton, bacteria, Protozoa, etc., but also as dead material such as decaying plants and animals. The organic matter varies greatly in particle size; phytoplankton cells, for example, range from about one to several hundred micra. The decaying material (detritus), of course, has no well-defined particle size and there is no sharp distinction between particulate and dissolved organic matter. Such a distinction, however often made, must therefore be a rather arbitrary one. "Particulate" material usually means material retained by paper or membrane filters with pore diameters of about one micron, whereas "dissolved" and "colloidal" matter passes through such filters. In this paper particulate organic matter is assumed to have a particle size of about one micron or more. The composition and the physical properties, such as particle size, of "dissolved" or "colloidal" organic matter are practically unknown. The latter is an important fraction, generally amounting to several times the particulate fraction. Values of dissolved organic nitrogen from various parts and depths of the sea range from 0.1 to 0.26 mg. per liter (Krogh and Keys, 1934, and von Brand and Rakestraw, 1941). Dissolved organic carbon is present in an amount of about 2.36 mg./liter. Assuming a mixture of carbohydrate and protein this would mean that about 1.5 mg. protein and 3.9 mg. carbohydrate are dissolved per liter of sea water (Krogh, 1934). The amount of particulate organic matter, living or dead, varies more than does that of dissolved matter. Some figures representative of coastal waters are given in Table I. Values from open sea and from great depths are smaller. However, the figures from coastal waters are of special interest to us because all determinations of filtration rates in filter feeders are gained in such areas.

It has often been discussed whether sufficient particulate organic matter is present in the water to cover the food requirements of filter feeders or whether dissolved or colloidal matter has to be taken into consideration as an additional resource. This discussion will not be reviewed here in detail. It should only be pointed out that the different approaches to the problem have led to contradictory results. One approach has been to estimate how much water should be filtered free of particulate food in order to meet the energy requirements of the animal in

[2] By "suitable technique" is meant a technique which does not significantly interfere with the normal filtering activity of the animal. Generally, even slight handling or disturbances of a filter feeder will reduce filtration rate. The values of water transport given in the present paper, as in other papers, are therefore more likely to be too low than too high.

question. Pütter (1909, 1914, 1925) made extensive studies on a variety of marine animals and concluded that the necessary filtration rates were beyond reasonable values. Fox and Coe (1943) made similar calculations for *Mytilus californianus*. They state that the quantity of micro-organisms present in the water does not provide enough nutriment for growth and metabolism of the mussel. They therefore conclude that about 5 mg. of organic matter, found by Krogh to be in solution in sea water, must be accessible to the mussels as food. On the other hand, direct attempts to demonstrate the importance as food of dissolved organic substances, either added or naturally occurring in the water, turned out negative (Krogh, 1931; Bond, 1933; and others).

It is of interest to repeat the calculations on the basis of our present knowledge of metabolic, growth and filtration rates, and to relate the results with the quantity of particulate organic matter which can be assumed to be present in the water.

TABLE I

Locality	Phytoplankton mg. organic matter per liter		Author	Remarks
	Range	Average		
English Channel	0.04–0.2 0.01–0.23	 0.06	Harvey, 1950 Atkins and Parke, 1951	Annual variation Annual variation
Baltic Sea	0.35		Krey, 1939	March
Long Island Sound	0.17–2.1 0.6 –2.8	0.59	Riley, 1941 Riley, Stommel and Bumpus, 1949	June–October June–September
Block Island Sound		0.38	Riley, personal communication	
Coastal water south of Cape Cod	0.14–0.63		Riley and Gorgy, 1948	July–September
Coastal water off La Jolla	0 –0.22	0.09	Graham, 1943	Annual variation

The calculations are based on a filtration rate of 15 liters for each ml. of oxygen uptake. One ml. of oxygen is required to combust about 0.8 mg. organic matter of mixed food. Fifteen liters of water should therefore contain at least 0.8 mg. organic matter which can be retained and utilized by the animals, or about 0.05 mg./liter. Obviously, organic matter which is used for growth must also be taken into consideration. The efficiency with which absorbed nutrients are utilized for growth varies with the age of the animals. In filter feeders such as lamellibranchs, gastropod veligers or copepods, up to about ⅔ of the energy absorbed can be used for growth (Harvey, 1950, and Jørgensen, 1952). Therefore, the actual food requirements for growth and respiration of the animals investigated probably does not exceed about 0.15 mg. utilizable organic matter per liter of water. The average figures for

phytoplankton (Table I) range from 0.06 mg. to 0.59 mg./liter, the lowest figure being found in the English Channel. Here, total organic particulate matter is, however, 1.6–1.8 mg./liter (Armstrong and Atkins, 1951). The values from La Jolla are perhaps too low because, in some instances, very little or no chlorophyll was measured despite rather high counts of chlorophyll-containing algae cells in the water. Thus at least during part of the year, particulate organic matter, phytoplankton and detritus, in the coastal waters is in excess of the amount needed for maintenance and growth of a number of filter feeders. The shortcoming of previous calculations is due mainly to an under-estimation of filtration rates, but also of the amount of particulate food in the sea.

It remains to be demonstrated that the filtering organs of the filter feeders are in fact able to retain what is generally measured as particulate organic matter in the sea, *i.e.*, material with a minimum particle size of about one micron. The efficiency of the filtering organs has been investigated in rather few species only. It was found that *Ciona intestinalis* almost completely retains one-micron particles. The same is probably true of other tunicates as well as sponges. In the lamellibranchs Mytilus, Ostrea and Pecten, the minimum particle size effectively retained varied from about one to a few micra (Jørgensen, 1949a, and Jørgensen and Goldberg, unpublished data). This probably also holds for many copepods (Ussing, 1938). Thus, most of the particulate organic matter in the sea is available to at least a considerable number of filter feeders.

Adjustment of food requirements to rate of food uptake. As mentioned above, changes in the concentration of food particles were not accompanied by changes in filtration rate.[3] The feeding rates therefore decrease with decreasing quantities of food in the water. Perhaps the reduction in metabolic rate to be observed in the course of the experiments with Molgula and other filter feeders can be interpreted as an adaptation to poor feeding conditions. Pütter (1914) observed a similar decrease in oxygen uptake in the sponge Suberites, and it is worth mentioning that v. Brand *et al.* (1948) found striking reductions (up to 80 per cent) in metabolic rates during starvation in a number of pulmonate gastropods.

However, more information is needed before we can obtain a clear picture of the interdependence between rate of filtration, metabolism, growth of different filter feeders and the concentration of food particles in the surrounding water.

Summary

The oyster, *Ostrea virginica*, and the ascidians *Ciona intestinalis* and *Molgula manhattensis* filter about 10 to 20 liters of water for each milliliter of oxygen consumed. It has been demonstrated that this filtration rate is probably great enough to provide the animals with sufficient amounts of particulate food for maintenance and growth.

LITERATURE CITED

Armstrong, F. A. J., and W. R. G. Atkins, 1951. The suspended matter of sea water. *J. Mar. Biol. Assoc.*, **29**: 139–144.

Atkins, W. R. G., and M. Parke, 1951. Seasonal changes in the phytoplankton as indicated by chlorophyll estimations. *J. Mar. Biol. Assoc.*, **29**: 609–618.

[3] It is unknown whether a slow adaptation of filtration rate to different concentrations of food particles can take place.

BOND, R. M., 1933. A contribution to the study of the natural food-cycle in aquatic environments. *Bull. Bingham Ocean. Coll., Art. IV*, **4**: 1–89.

VON BRAND, T., AND N. W. RAKESTRAW, 1941. The determination of dissolved organic nitrogen in sea water. *J. Mar. Res.*, **4**: 76–80.

VON BRAND, T., M. O. NOLAN AND E. R. MANN, 1948. Observations on the respiration of *Australorbis glabratus* and some other aquatic snails. *Biol. Bull.*, **95**: 199–213.

FOX, D. L., AND W. R. COE, 1943. Biology of the California sea-mussel (*Mytilus californianus*). II. Nutrition, metabolism, growth and calcium deposition. *J. Exp. Zool.*, **93**: 205–249.

GAULD, D. T., 1951. The grazing rate of planktonic copepods. *J. Mar. Biol. Assoc.*, **29**: 695–706.

GRAHAM, H. W., 1943. Chlorophyll-content of marine plankton. *J. Mar. Res.*, **5**: 153–160.

HARVEY, H. W., 1950. On the production of living matter in the sea of Plymouth. *J. Mar. Biol. Assoc.*, **29**: 97–138.

JØRGENSEN, C. B., 1949a. The rate of feeding by Mytilus in different kinds of suspension. *J. Mar. Biol. Assoc.*, **28**: 333–344.

JØRGENSEN, C. B., 1949b. Feeding-rates of sponges, lamellibranchs and ascidians. *Nature*, **163**: 912.

JØRGENSEN, C. B., 1952. Energetic efficiency of growth in *Mytilus edulis* and two gastropod veligers. *Nature*. (In press.)

KREY, J., 1939. Bestimmung des Chlorophylls. *J. Cons. Intern. Explor. Mer.*, **14**: 201–209.

KROGH, A., 1931. Dissolved substances as food of aquatic organisms. *Cons. Perm. Intern. Explor. Mer. Rap. Proc. Verb.*, **75**: 7–36.

KROGH, A., 1934. Conditions of life in the ocean. *Ecol. Monog.*, **4**: 421–429.

KROGH, A., AND A. KEYS, 1934. Methods for the determination of dissolved organic carbon and nitrogen in sea water. *Biol. Bull.*, **67**: 132–144.

LOOSANOFF, V. L., AND J. B. ENGLE, 1947. Effect of different concentrations of microorganisms on the feeding of oysters (*O. virginica*). *U. S. Fish and Wildlife Serv. Bull.*, **51**: 31–57.

PÜTTER, A., 1909. Die Ernährung der Wassertiere und der Stoffhaushalt der Gewässer. Fischer, Jena.

PÜTTER, A., 1914. Der Stoffwechsel der Kieselschwämme. *Zeitschr. allg. Physiol.*, **16**: 65–114.

PÜTTER, A., 1925. Die Ernährung der Copepoden. *Arch. Hydrobiol.*, **15**: 70.

RAYMONT, J. E. G., AND D. T. GAULD, 1951. The respiration of some planktonic copepods. *J. Mar. Biol. Assoc.*, **29**: 681–693.

RILEY, G. A., 1941. Plankton studies III. Long Island Sound. *Bull. Bingham Ocean. Coll., Art. III*, **7**: 1–93.

RILEY, G. A., AND S. GORGY, 1948. Quantitative studies of summer plankton populations of the Western North Atlantic. *J. Mar. Res.*, **7**: 100–121.

RILEY, G. A., H. STOMMEL AND D. F BUMPUS, 1949. Quantitative ecology of the plankton of the Western North Atlantic. *Bull. Bingham Ocean. Coll., Art. III*, **12**: 1–169.

USSING, H. H., 1938. The biology of some important plankton animals in the fjords of East Greenland. Medd. om Grønland, **100**: 1–108.

APPLICATION OF THE MOST PROBABLE NUMBER METHOD TO DETERMINE HEAT SENSITIVITY OF PROTOZOA

JOHN B. LOEFER AND ROY B. MEFFERD, JR.[1]

Southwest Foundation for Research and Education, and Trinity University, San Antonio, Texas

In order to determine the deleterious effect of specific agents or treatments on populations of micro-organisms, it is desirable to employ an end-point dilution method to determine number of viable survivors. Plating procedures have not been suitable for use with protozoa, except in the case of certain non-motile and pigmented forms. Lack of a suitable method has hampered studies on the sensitivity of protozoa to specific chemical substances or physical treatments. The most probable number method, frequently used by bacteriologists, was adapted for use in a study on heat sensitivity of Tetrahymena, and the results are described below.

Material and Methods

The ciliated protozoan, *T. pyriformis*, strain WB, as designated previously (Loefer, 1952) and used in an earlier investigation (Loefer and Matney, 1952), was employed in this study. Ingredients of the medium were: Bacto-Casitone, 2%; Bacto Yeast Extract, 0.25%; and salts in mg. % as follows: NaCl, 20; $CaSO_4$, 5; $MgSO_4$, 2.5; KNO_3, 20; KH_2PO_4, 10; K_2HPO_4, 10; $FeCl_3$, 0.5; pH, 7.1–7.3. Cultures in their log phase were used for all determinations. Exposure to specific temperatures was effected in 22 × 175 mm. Pyrex tubes in a constant temperature water bath (± 0.01° C.). A concentrated suspension of ciliates (0.1 ml.) was introduced into pre-heated media (20.0 ml.) and aliquots were removed aseptically at specific time intervals as indicated and transferred to cool (25° C.) dilution bottles containing 0.1% Bacto-Casitone plus inorganic salt solution, so as to obtain series of ten-fold dilutions. Suitable buffering and poising of the dilution fluid by the addition of a low concentration of regular medium is desirable. Additional advantages in the use of 0.1% medium for dilution are that the dilution bottles may be pretested for sterility prior to use, and also, since the bottles foam slightly when shaken, errors during the dilution process are avoided.

Most probable number technique

Five tubes containing approximately 10 ml. of regular medium (penicillin or streptomycin may be added to inhibit bacterial growth, if technique is not rigidly aseptic) were then inoculated with one ml. from each of the above-mentioned dilutions. After a suitable incubation period (96 hours), the tubes were checked for positive growth.

The most probable number of organisms in the original culture was found by reference to the data shown in Table I. The number of positive tubes (from each

[1] Post-doctoral Fellow in Cancer Research of the Damon Runyon Memorial Fund.

TABLE I

Most probable number per milliliter of sample, planting 5 portions in each of 3 dilutions in geometric series. (Modified from Hoskins, 1934)

(1) x			(2)	(1) x			(2)	(1) x			(2)	(1) x			(2)	(1) x			(2)	(1) x			(2)
1	.1	.01		1	.1	.01		1	.1	.01		1	.1	.01		1	.1	.01		1	.1	.01	
0	0	0		1	0	0	2	2	0	0	4.5	3	0	0	7.8	4	0	0	13	5	0	0	23
0	0	1	1.8	1	0	1	4	2	0	1	6.8	3	0	1	11	4	0	1	17	5	0	1	31
0	0	2	3.6	1	0	2	6	2	0	2	9.1	3	0	2	13	4	0	2	21	5	0	2	43
0	0	3	5.4	1	0	3	8	2	0	3	12	3	0	3	16	4	0	3	25	5	0	3	58
0	0	4	7.2	1	0	4	10	2	0	4	14	3	0	4	20	4	0	4	30	5	0	4	76
0	0	5	9	1	0	5	12	2	0	5	16	3	0	5	23	4	0	5	36	5	0	5	95
0	1	0	1.8	1	1	0	4	2	1	0	6.8	3	1	0	11	4	1	0	17	5	1	0	33
0	1	1	3.6	1	1	1	6.1	2	1	1	9.2	3	1	1	14	4	1	1	21	5	1	1	46
0	1	2	5.5	1	1	2	8.1	2	1	2	12	3	1	2	17	4	1	2	26	5	1	2	64
0	1	3	7.3	1	1	3	10	2	1	3	14	3	1	3	20	4	1	3	31	5	1	3	84
0	1	4	9.1	1	1	4	12	2	1	4	17	3	1	4	23	4	1	4	36	5	1	4	110
0	1	5	11	1	1	5	14	2	1	5	19	3	1	5	27	4	1	5	42	5	1	5	130
0	2	0	3.7	1	2	0	6.1	2	2	0	9.3	3	2	0	14	4	2	0	22	5	2	0	49
0	2	1	5.5	1	2	1	8.2	2	2	1	12	3	2	1	17	4	2	1	26	5	2	1	70
0	2	2	7.4	1	2	2	10	2	2	2	14	3	2	2	20	4	2	2	32	5	2	2	95
0	2	3	9.2	1	2	3	12	2	2	3	17	3	2	3	24	4	2	3	38	5	2	3	120
0	2	4	11	1	2	4	15	2	2	4	19	3	2	4	27	4	2	4	44	5	2	4	150
0	2	5	13	1	2	5	17	2	2	5	22	3	2	5	31	4	2	5	50	5	2	5	180
0	3	0	5.6	1	3	0	8.3	2	3	0	12	3	3	0	17	4	3	0	27	5	3	0	79
0	3	1	7.4	1	3	1	10	2	3	1	14	3	3	1	21	4	3	1	33	5	3	1	110
0	3	2	9.3	1	3	2	13	2	3	2	17	3	3	2	24	4	3	2	39	5	3	2	140
0	3	3	11	1	3	3	15	2	3	3	20	3	3	3	28	4	3	3	45	5	3	3	180
0	3	4	13	1	3	4	17	2	3	4	22	3	3	4	31	4	3	4	52	5	3	4	210
0	3	5	15	1	3	5	19	2	3	5	25	3	3	5	35	4	3	5	59	5	3	5	250
0	4	0	7.5	1	4	0	11	2	4	0	15	3	4	0	21	4	4	0	34	5	4	0	130
0	4	1	9.4	1	4	1	13	2	4	1	17	3	4	1	24	4	4	1	40	5	4	1	170
0	4	2	11	1	4	2	15	2	4	2	20	3	4	2	28	4	4	2	47	5	4	2	220
0	4	3	13	1	4	3	17	2	4	3	23	3	4	3	32	4	4	3	54	5	4	3	280
0	4	4	15	1	4	4	19	2	4	4	25	3	4	4	36	4	4	4	62	5	4	4	350
0	4	5	17	1	4	5	22	2	4	5	28	3	4	5	40	4	4	5	69	5	4	5	430
0	5	0	9.4	1	5	0	13	2	5	0	17	3	5	0	25	4	5	0	41	5	5	0	240
0	5	1	11	1	5	1	15	2	5	1	20	3	5	1	29	4	5	1	48	5	5	1	350
0	5	2	13	1	5	2	17	2	5	2	23	3	5	2	32	4	5	2	56	5	5	2	540
0	5	3	15	1	5	3	19	2	5	3	26	3	5	3	37	4	5	3	64	5	5	3	920
0	5	4	17	1	5	4	22	2	5	4	29	3	5	4	41	4	5	4	72	5	5	4	1600
0	5	5	19	1	5	5	24	2	5	5	32	3	5	5	45	4	5	5	81	–	–	–	——

(1) Significant number: positive tubes* with dilutions 1x, 0.1x and 0.01x.

(2) Most probable number per milliliter.

* The dilution "x," used in the tubes, representing the first digit in the significant number, is corrected as follows:

Where x is a dilution of	*Multiply MPN value by*
1:10	1
1:100	10
1:1000	100
etc.	

group of five) in the entire series of geometric dilutions may be represented as a multi-digit number, *e.g.*, 5 5 5 4 3 1, in which the first digit represents the number of positive tubes from the 1 : 10 dilution, and the last number from the 1 : 1,000,000 solution. The number to be used for reference to the table, *i.e.*, the significant number, is 5 4 3 1. The first digit of the significant number represents the number of tubes of the highest dilution from which all were positive, or the lowest dilution used, in the event less than 5 are positive. Since the significant number must consist of three digits, any fourth digit is added to the third, and the example given above now becomes 5 4 4. Reference to column 1 of the table indicates a most probable number of 350. Since the dilution for the first digit of the significant number was 1 : 1000, the most probable number is multiplied by the correction factor indicated in the table. In the illustration given, we obtain 35,000 organisms per milliliter as the most probable number.

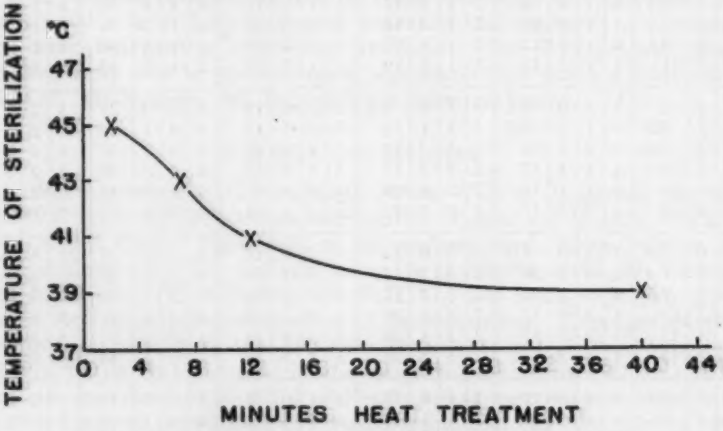


FIGURE 1. Thermal death time for *Tetrahymena pyriformis* WB.

Correlations were made between direct microscopic counts (Hall, Johnson and Loefer, 1935) and most probable number determinations. Average results of either did not vary by more than 5% when replicate determinations were made. The slightly higher count observed consistently in direct counts is readily explicable since any culture contains a certain number of non-viable organisms which will not be detected in the most probable number determination.

EXPERIMENTAL

Viable survivors at specified time intervals were determined following treatment of cultures at 39, 41, 43 and 45° C. In addition, thermal death time at each temperature was determined and these data are presented in Figure 1. The sharp increase in the thermal biological effect as the temperature is raised is due to the high Q_{10} of protein denaturation. At 37° C., several hours were required to obtain a sterile culture.

Although survivor curves were obtained at each of the temperatures tested, those following treatment at 39 and 45° C. are presented as being typical (Fig. 2). As opposed to the case following treatment with ultraviolet radiation (Mefferd and Loefer, 1952), there was a close correlation between loss of motility and loss of viability. In all cases there is a period of relatively slow death rate preceding the period of most rapid killing. A part of this lag undoubtedly is a result of the time required to raise individual cells to the temperature of the water bath, but this explains only a part of the effect. The most logical explanation remains that given by Rahn (1931) which assumes that there is more than one molecule which must be destroyed in order to cause death of the cell. A possibility which must be considered

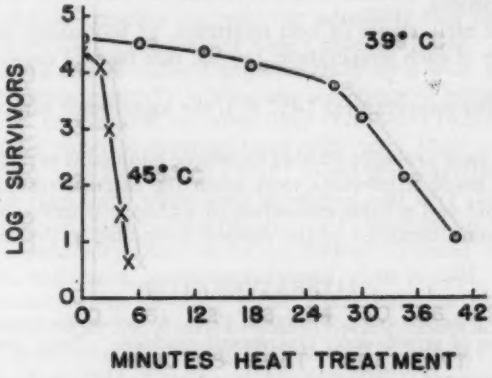


FIGURE 2. Survivor curves for cultures of *Tetrahymena pyriformis* WB subjected to 39 and 45° C.

is that the protozoan population varies widely in the thermal resistance of its members (Jahn, 1933). When large inocula are employed, it is not difficult to select a population of cells with markedly enhanced viability at high temperatures (Mefferd and Campbell, 1952). The authors have, by such techniques, obtained strains of *T. pyriformis* which are capable of growth at 37° C. and even higher. However, in the usual population of a few million cells, the portion which is heat resistant would only negligibly affect the survival curve.

Our data agree generally with those reported by Jahn (1933) for *Euglena gracilis*, and the slight difference may be explained by two major modifications in our technique which varied from his, *viz.*, the introduction of a very small volume of concentrated organisms into a large volume of pre-heated medium, and the quantitative determination of viable cells utilizing a technique (most probable number) which is dependent only upon whether or not an aliquot contains one or more viable cells. This eliminates the many factors which may influence methods based upon comparative growth rates, such as unequalized population densities and the difference in nutritional supplementation of the media between a culture in which no organisms are killed and another in which many have been killed and lysed. The importance of either of these factors is evident.

It is important for the successful application of the technique that incubation of tubes be continued for an extended period of as many as six days or so. In each series there are tubes which contain as few as one organism. Starting from this small inoculum, considerable time is required to achieve a detectable number of cells in the tube. A small quantity of a suitable antibiotic in the dilution and growth media is a distinct advantage in inhibiting chance bacterial contaminants.

Summary

1. Utilizing the most probable number technique, thermal death time and viable survivor curves over a series of temperatures (39, 41, 43 and 45° C.) were determined for Tetrahymena.

2. During the early stages of heat treatment, an increasing death rate was observed in the case of each temperature, but the rate became constant (logarithmic) after a time.

3. At the higher temperatures (45° C.), the logarithmic rate was achieved in a very short time.

4. Use of the most probable number technique eliminates several of the criticisms which have been made of previous work upon the thermal sensitivity of protozoa, and enables a rapid and reliable estimation of viable cells following heat treatment. The advantages and limitations of the method as applied to protozoa are discussed.

LITERATURE CITED

Hall, R. P., D. F. Johnson and J. B. Loefer, 1935. A method for counting protozoa in the measurement of growth under experimental conditions. *Trans. Amer. Micr. Soc.*, **54**: 298–300.

Hoskins, J. K., 1934. Most probable numbers for evaluation of coli-aerogenes. Tests by fermentation tube method. *Public Health Rep.*, **49**: 393–405.

Jahn, T. L., 1933. Studies on the physiology of the euglenoid flagellates. IV. The thermal death time of *Euglena gracilis* Klebs. *Arch. f. Protisten.*, **79**: 249–262.

Loefer, J. B., 1952. Some observations on the size of Tetrahymena. *J. Morph.*, **90**: 407–414.

Loefer, J. B., and T. S. Matney, 1952. Growth inhibition of free-living protozoa by actidione. *Physiol. Zool.*, **25**: 272–276.

Mefferd, R. B., Jr., and L. L. Campbell, Jr., 1952. Studies on a thermophilic variant of *Bacillus globigii*. *Tex. Rep. Biol. Med.*, **10**: 419–420.

Mefferd, R. B., Jr., and J. B. Loefer, 1952. Lethality of ultraviolet radiation for *Tetrahymena pyriformis*. *Proc. Soc. Protozool.*, **3**: 17.

Rahn, O., 1931. The order of death of organisms larger than bacteria. *J. Gen. Physiol.*, **14**: 315–337.

FACTORS INFLUENCING METAMORPHOSIS OF BUGULA LARVAE

WILLIAM F. LYNCH

St. Ambrose College, Davenport, Iowa, and the Marine Biological Laboratory, Woods Hole, Mass.

A review of the literature on experimentally induced metamorphosis in invertebrates and in sessile tunicates suggests the possibility that essentially similar mechanisms may be involved in animals of widely different phyla. As in parthenogenesis, metamorphosis can be induced experimentally by a wide variety of conditions and chemical agents, generally lacking any semblance of specificity; and most of these seem to be unfavorable to larval life. Huxley (1928) effected metamorphosis in Echinus plutei by treating them with very dilute concentrations of $HgCl_2$ ($M/2{,}000{,}000$), and Runnström (1917) found that starvation of the larvae, over-ripeness of the eggs from which they developed and very dilute solutions of $ZnSO_4$ in sea water would cause dedifferentiation in Echinus larvae. Grave (1935) found that ascidian metamorphosis can be accelerated by many agents including CO_2-free sea water, lactic acid, precipitates formed when NaOH, KOH or $BaCl_2$ are added to sea water, whole pituitary gland, tissue extracts of ascidian mantle and endostyle and of the foot and mantle of snails; these extracts are highly specific in nature. Other promoters of ascidian metamorphosis include: copper (Grave, 1941; Bertholf and Mast, 1944; Glaser and Anslow, 1949), concentrated sea water (Bertholf and Mast, 1944), thyroid extract (Weiss, 1928) and whole gland (Grave, 1935), methylene blue (Bertholf, 1945; Zhinkin, 1938), neutral red (Bradway, 1936; Zhinkin, 1938), brilliant cresyl blue (Zhinkin, 1938), Janus green (Bertholf and Mast, 1944), a brief exposure to distilled water (Bertholf, 1945), inorganic iodine but not thyroxine (Bradway, 1936), "conditioned sea water" (Grave and Nicoll, 1936; Grave, 1944; Bertholf and Mast, 1944) and isotonic NaCl (Bradway, 1936). (Grave, 1935, had found that the addition of sodium and potassium ions to sea water had no effect on metamorphosis; Bradway, however, used a different technique with an optimum amount of exposure and used isotonic NaCl instead of sea water to which the ions had been added.) Metamorphosis can be induced in the larva of the oyster, *Ostrea virginica,* by copper (Prytherch, 1934; Armstrong and Miall, 1945) and by a certain optimum of salinity (Prytherch, 1934); but neither the cations nor anions of sea water nor the metal carbonates of Fe, Al, Ag, Ni, Ba, Pb and Sn have any effect according to Prytherch (1934). Copper, hypertonic sea water and heat accelerate metamorphosis in the bryozoan, *Bugula flabellata* (Lynch, 1949a).

Since fluids in which proteolytic digestion has occurred bring about a high concentration of neutral red from very dilute solutions, whereas coelomic fluids do not take the stain, Koehring (1930, 1931) proposed that the azo radical of the dye combines with and activates proteolytic enzymes and that neutral red staining can be used as an index of the site and intensity of enzyme activity. She found the dye

effective in causing parthenogenesis in starfish eggs. Neutral red in concentrations of 1 : 50,000 parts (Bradway, 1936) and of 1 : 200,000–1 : 800,000 parts in sea water (Zhinkin, 1938) accelerates metamorphosis in ascidian larvae, presumably by activating autolytic enzymes involved in resorption of the tail. Zhinkin (1938) found methylene blue also effective in approximately the same concentrations. Since some agents have the common effect of inducing precocious fixation in animals of widely different phyla, it was thought that the two vital dyes might accelerate metamorphosis in *Bugula* larvae.

The present paper concerns a re-investigation of the role played by the ions of sea water under more rigidly controlled conditions of light, osmotic pressure and the pH of the medium than in former experiments, and of the effects of media containing only three or two ions and combinations of these with vital dyes; the work was undertaken since it was considered that certain factors may act antagonistically to accelerators of metamorphosis whereas others may synergize their effects. This supposition was verified, sometimes in a striking manner. The larvae were secured by means already described (Lynch, 1947) and the methods used will be presented in the separate sections. Casters and syracuse dishes were used for most observations with a binocular microscope and these were kept covered to prevent shifting of the pH; when controls were used they were kept at the same number of foot candles of illumination (Weston photometer) as the experimental dishes, since light has an accelerating effect on metamorphosis.

Results

An excess of the chlorides of Na, K, Ca and Mg in sea water. General observations on the effects of concentrating these chlorides in sea water have already been published (Lynch, 1947, 1949a). During the summers of 1951 and 1952 experiments on the effects of an excess of each of the four chlorides were carried out by using only isotonic solutions in sea water, in proportions of 80/20, 50/50 and 20/80 cc. For magnesium and potassium the results were essentially the same as those reported (Lynch, 1949a). Metamorphosis was inhibited at a pH of 7.1, that of the original mixtures, and at 8.0 (raised by NaOH in magnesium mixtures and by

Table I

The effects of an excess of NaCl on the motility of larvae

Larvae in 80 cc. isotonic NaCl/20 cc. sea water pH = 8.1 (NaOH)				Larvae in normal sea water on the same day			
No. of larvae	No. active in hours			No. of larvae	No. active in hours		
	5	12	24		5	12	24
70	50	0	0	34	16	5	1
115	47	3	0	37	22	2	1
48	16	0	0	59	24	7	1
8	5	0	0	29	14	4	1
12	8	0	0	12	5	4	0
253		3 = 1.1%		171		22 = 12.2%	

KOH in those containing an excess of potassium). The prolonged swimming, formerly observed in the magnesium mixtures, was evidently due to the hydronium ion, since it does not occur at a pH of 8.0 and experiments with sea water acidulated by HCl have shown a considerable lengthening of the natant phase. Since the almost immediate metamorphosis observed in the 50/50 mixtures of 1 N NaCl and $CaCl_2$ could have been effected by the hypertonicity of these solutions, especially the former, the experiments were repeated with isotonic NaCl and $CaCl_2$. A high osmotic pressure was undoubtedly the cause of accelerated metamorphosis in the case of sodium chloride, for proportions of 80 cc. of isotonic NaCl/20 cc. of sea water did not hasten setting; in fact, metamorphosis did not occur in some experiments. In general, only larvae that attached to the surface or to the photonegative

TABLE II

The normal duration of the natatory phase in sea water

No. of larvae	No. inactive in hrs.			
	4	8	12	24
73	71	—	73 (100%)	73
39	28	—	33 (87%)	39
56	21	—	35 (62%)	56
86	7	28	38 (44%)	73
138	84	102	123 (90%)	129
109	4	14	66 (60%)	109
34	—	—	29 (85%)	33
37	—	—	35 (94%)	36
59	—	—	51 (86)	58
29	—	23	25 (86)	28
12	—	3	8 (66)	12
14	—	10	13 (93)	14
686			529	

Average number inactive at 12 hrs. = 77%. Sigma = 16%.

side of the dishes containing an excess of NaCl metamorphosed normally. The natatory period, however, was abbreviated as reported for the normal solutions (see Table I). On the other hand 80 cc. of isotonic $CaCl_2$/20 cc. of sea water gave results essentially identical with those described for normal solutions, but metamorphosis occurred almost immediately only when the pH had been raised to 8.0 ($CaCO_3$, NaOH, KOH and borate buffers gave similar results). The reverse proportions, however (20 cc. of $CaCl_2$/80 cc. of sea water), failed to show any statistically significant difference between the behavior of larvae in these media and that of the controls. The cause of the observed prolongation of the natatory period in solutions having the same proportions of sea water and 1 N $CaCl_2$ remains obscure. It seems unlikely that it was mere coincidence, for larvae are rarely active at 24 hours in sea water (*cf.*, Table II), unless the pH is lowered to about 6.5, and experiments with isotonic solutions gave no indication of such a drop in pH. (Table III shows that many larvae are active at 24 hours in these solutions at a low pH.) These studies have also shown that hypertonic solutions ac-

celerate metamorphosis, regardless of whether the osmotic pressure is raised by ions of salts or by molecules of sugar. In former experiments with solutions made hypertonic by adding sucrose to sea water, the osmotic pressure had not been raised sufficiently high to give accelerating effects. (Hastening of setting does not occur if the freezing point depression is much below 2.73.)

TABLE III

The natatory period of larvae in 80 cc. of sea water and 20 cc. $CaCl_2$ at pH = 6.4

Experimental medium		Controls (same days; pH = 6.4)	
No. larvae	No. active at 24 hrs.	No. larvae	No. active at 24 hrs.
84	17	88	2
81	6	44	1
76	6	89	5
185	25	58	0
145	41	64	4
101	25	69	0
41	4	12	2
31	6	16	2
744	130 = 17.4%	440	16 = 3.6%

Three-ion combinations. These mixtures were made by omitting one of the ions from the following proportions used by LeFevre (1948) in making artificial sea water from isotonic solutions: 500 cc. of NaCl, 10 cc. of KCl, 15 cc. of $CaCl_2$, 2 cc. of $NaHCO_3$, 40 cc. of $MgCl_2$ and 15 cc. of $MgSO_4$. Van't Hoff's (beta) solutions, however, were used for calcium-free media and $MgSO_4$ was omitted in all solutions containing calcium (because of precipitation) and its place was taken by 15 cc. of $MgCl_2$. Experiments were performed with solutions in which the missing ions were replaced by an equal volume of isotonic sucrose and with mixtures without sugar but having the three ions in the same ratio as in artificial sea water. The presence or absence of sucrose seemed to have no effect, although it was found that metamorphosis was inhibited in solutions containing 80 cc. of sucrose/20 cc. of sea water at a pH of 7.8 (glycine buffer). This procedure of making three-ion media is open to the objections summarized by Heilbrunn (1943, p. 455) and the absence of sulphate may have had an independent effect. (For the effects of an absence of sulphate on echinoderm animal-halves treated with lithium, see Needham, 1950, p. 492.) Larvae were placed for a minute or two in 15 cc. of the three-ion medium before being transferred to another dish of the same solution. Generally three or four transfers were made to minimize contamination during seeding with larvae. Nevertheless, the term "ion-deficient media" is preferable to "ion-free" since a slight amount of contamination is unavoidable. The pH was raised to 7.8–8.0 by NaOH and KOH in all experiments.

In all except Mg-free solutions metamorphosis failed to occur. Results were quite similar in Na-free, Ca-free and K-free media. In all of these the second stage of metamorphosis, migration of tissue from the pallial furrow and subsequent pushing of the ciliated covering towards the basal end of the larvae, could

be observed; but the first stage, eversion of the internal sac, failed to occur. This may not be quite true of larvae in Na-free media, for the internal sac protruded from the organisms while swimming but failed to attach them to the substrate. A great elongation of the apical end accompanied by a migration of ciliated covering towards the basal region caused the long axis of the larvae to be lengthened and a prominent bulge to appear in the middle where the ciliated tissue had accumulated in the Na-free media. After an hour and a half the larvae became spherical again and the cilia seemed to disintegrate eventually. Organisms in Ca-free van't Hoff's solution failed to show any protrusion of the internal sac and eventually shed their outer ciliated coverings into the surrounding medium exactly like those placed in an excess of $MgCl_2$ (Lynch, 1949a). The larvae exhibited neither the usual light reactions nor the back-and-forth swimming of the controls. Highly concentrated in the center of the dish, these larvae confined their movements to narrow circumscribed areas, consisting of either circular counterclockwise swimming or rotation about a fixed point. By 6–8 hours these movements had ceased but ciliary action continued. (The latter was most prolonged at a pH of 6.3.) By 24 hours cytolysis was pronounced in larvae left in this medium. Motility was much better preserved in Ca-free than in Na-free media, for in the latter there was scarcely any movement at 1½ hours. In K-free media the behavior was essentially like that described above, except that adhesive fluid was gradually shed from the internal sac and congealed behind the larvae while they were still swimming. (This phenomenon is not at all uncommon in experimental media, for it occurs in sea water when the pH is lowered to 7.0 by McIlvaine buffer and in other cases to be described.) Again, in K-free solutions the behavior of the larvae strikingly resembled that of organisms placed in an excess of magnesium chloride.

Metamorphosis occurred only in Mg-free media within a pH range of 7.4 to 8.0 (raised by NaOH in the latter). Setting was considerably accelerated, having been completed in nearly all larvae within a period of 30 minutes to 3 hours. (The normal duration of the natatory period of larvae in sea water is variable, sometimes occurring within two hours and occasionally enduring in a few larvae for 24 hours; *cf.*, Table II. Several trials have shown that by 12 hours 77% ± 5 have metamorphosed.) When metamorphosed larvae were transferred from the Mg-free media to sea water after 3 hours they reached the polypide stage by 24 hours; those left in the original solutions elongated somewhat but did not reach the condition at which stolons are organized into tripod-shaped structures for anchorage, which usually occurs about 8 hours after metamorphosis. All adhered rigidly to the substrate. The first reaction of larvae placed in Mg-free media was one of complete quiescence; then natatory movements were gradually resumed. Many of the organisms emitted small amounts of adhesive material from the internal sac shortly after contact with solutions at a pH of 7.4, that of the original mixture. A deficiency or absence of magnesium had an effect opposite to that of an excess of the ion.

Two-ion combinations. Normal motility of bryozoan larvae can be maintained for an amazingly long time when only two ions are present. (1) In 20 cc. of isotonic NaCl/10 cc. of $MgCl_2$ (pH raised to 8.0 by NaOH) the larvae remained motile for 6–8 hours after three transfers. Again the ciliated covering migrated towards the basal region of the larvae and these organisms lost their reaction to

light, gathering in enormous numbers in the center of the dish. In fact, an unusual protrusion of the apical organ and migration of ciliated covering towards the basal end, or at least the equatorial region, of the larvae seems to be a characteristic reaction when any one of the four ions of sea water is missing from the media. (2) When the proportions were reversed (20 cc. of $MgCl_2$/10 cc. of NaCl at a pH of 8.0) motility was maintained for as long as 10 hours and typical magnesium effects were observed: absence of light reactions and emission of tissue from the pallial furrow. (3) Similar phenomena occurred in 15 cc. of $CaCl_2$/55 cc. of $MgCl_2$ and motility endured for at least 4 hours after three transfers. This is surprising in view of the fact that natatory movements are extremely feeble by 1½ hours in three-ion Na-free solutions. Evidently the absence of potassium favors ciliary action, for it is more injurious to the cilia of the larvae than any other salt except copper when an excess is added to sea water. Likewise magnesium seems to be necessary for natatory movements of long duration, for in Mg-free three-ion combinations only feeble movements persisted for three hours; in solutions of 20 cc. NaCl/10 cc. of $CaCl_2$ (pH = 8.0 by NaOH) the larvae became immobilized almost immediately and began rotating about a fixed point, a movement that immediately precedes metamorphosis under normal conditions, by means of their vibratile flagella. (The latter form a tuft of long hair-like structures at the apical end of the median furrow or lateral groove; movements of these flagella persist long after ciliary action has ceased.) Great care must be taken to separate ionic effects from those due to the pH of the medium, since larvae in sea water acidulated to a pH of 5.8–6.0 (HCl) may continue swimming for 54 hours or longer, even though the pH will have shifted to 7.0 during 24 hours. Metamorphosis did not occur in any of the two-ion combinations after two transfers, although an atypical kind of setting was observed in 20 cc. of NaCl/10 cc. of $CaCl_2$. Although the larvae did elongate somewhat when removed to sea water after a short exposure to this medium, repeated observations could not determine whether this was a true metamorphosis or not. It is difficult to distinguish the chaotic condition of metamorphosis from rapid cytolysis that generally occurs under these conditions. Even the small amount of sea water added during seeding allowed normal setting in 20 cc. of $MgCl_2$/10 cc. of NaCl; larvae on the surface formed zooids with tripod-like stolons for anchorage. (It is interesting to note in passing that this is characteristic of *B. flabellata,* whereas *B. turrita* always develops four symmetrically arranged stolons, each branching dichotomously at its distal end into two branches.)

Neutral red in sea water. In the following experiments one drop of 0.1% aqueous solution of neutral red was added to 10, 30, 50, 100, 150 and 200 cc. of sea water (range of concentration = 0.001–0.00005%; 1 : 100,000–1 : 2,000,000 parts). The pH of the highest concentration was 7.8–8.0 and the freezing point depression was 1.71. Both *B. turrita* and *B. flabellata* were employed during the summer of 1951, but only the latter in 1952; the controls and the experimental organisms were always of the same species and dishes containing them were placed in diffuse light of 75 foot-candles. Temperatures varied from 25–29° C. Larvae were transferred once from a test solution to another of equal concentration to minimize the dilution which occurred during seeding.

Two striking effects were caused by the neutral red media. First, the photonegative response, which generally takes place in sea water at two–three hours,

had set in by an hour in all solutions; and in most cases the change from positive to negative phototropism began within ten minutes after placement of the larvae. These organisms were also more intensely negative than the controls and frequently attached *en masse* at a spot on the periphery of the dish farthest from the source of light. Secondly, metamorphosis was accelerated in solutions containing one drop/10, 50 and 100 cc. of sea water (concentrations = 0.001–0.0001%), but dilutions greater than 0.0001% had no appreciable effect. The degree of acceleration appeared to be somewhat roughly proportional to the concentration; except in media containing one drop/10 cc., the duration of the natant phase varied with that of the controls, being shortest on days when the latter metamorphosed more quickly. In solutions containing one drop/10 cc. of sea water, the free-swimming period was about an hour with very little deviation on either side. In the other solutions found to be effective the extreme duration of the natatory period was four hours; at this time only a few larvae were still active, whereas the majority of the controls were motile at 12 hours.

Subsequent development depended upon whether the larvae were left in the neutral red solution or transferred to sea water after metamorphosis. The results of various concentrations may be summarized as follows: (1) Larvae in the most concentrated solution (1/10) had an elongation equal to that of the controls by 24 hours, but actual development was better when the larvae were transferred to sea water after metamorphosis had been induced precociously, for in the latter case differentiation sometimes exceeded that of the controls. In one experiment in which larvae were allowed to remain in the neutral red solution, about ⅔ had become decidedly stained and at 24 hours these had formed normal zooids as well advanced as the controls except that polypide formation was somewhat inferior; the other ⅓ were free from dye and had not metamorphosed. (2) In solutions of one drop/50 cc. of sea water development was inferior to that of the controls in three out of four experiments in which the larvae remained in the neutral red solution for 24 hours; differentiation was normal, however, when the organisms were removed to sea water after metamorphosis. In at least some cases of poor zooid formation the effect can be attributed to a loss of fluid from the internal sac while the larvae were still swimming; this fluid congealed to form threads which resembled those that had been observed when organisms were placed in sea water maintained at a pH of 7.0 by potassium phosphate McIlvaine buffers, except that the threads formed a network in the latter. Since the holdfast material forms the zooecial wall of the zooid, its deficiency would result in poor development. (For the contribution of larval parts to the adult organism the monograph of Corrêa (1948) on the embryology of *Bugula flabellata* should be consulted.) (3) In dilutions as great as one drop/100 cc. development was equal to that of the controls even when the larvae were not transferred to sea water. (4) In solutions found to be ineffective in appreciably accelerating metamorphosis, development was variable (as it often was in the controls), and in general it seemed to be no different from that of larvae in sea water.

Staining reactions of neutral red. The apical half of the pyriform larvae (containing the crown of rigid cilia) stained very lightly, whereas the basal half (containing the internal sac) stained heavily, the dye being concentrated in definite spots causing the larvae to have a mottled appearance. Staining was generally

pronounced within 20–30 minutes after immersion in the most concentrated media (one drop of neutral red/10 cc. of sea water and one drop/30 cc.). During the process of metamorphosis, material from the pallial furrow (lacking cilia) did not take the dye; and as this material migrated slowly to the basal end of the larvae, the dye gradually became concentrated in granular spots beneath it in this region. The ciliated covering, which is gradually pushed inside the metamorphosing larva by advancing material from the pallial furrow, did not take the stain. This could be determined under abnormal conditions (Ca-free solutions) in which the ciliated covering was shed into the surrounding medium. Holdfast material was likewise largely colorless except for a few granules. After metamorphosis had been completed, the zooecial wall of the first polypide was almost free from stain except for a few red granules scattered here and there but especially concentrated at the distal end where the lophophore would form later. The former body of the larva, located about a third of the distance between the attached end and the free lophophore region, was deeply stained. In later development the polypide, containing the digestive tract, was deeply stained within the zooecial wall. Some of the dye went into the bud for the second zooid of the colony, again concentrating at the tip where the lophophore would form. Buds for the third zooid generally remained free from stain. The tentacles as well as the stolons, except at the distal ends of the latter, were colorless.

Neutral red in three-ion combinations. These media were made as described above and one drop of neutral red/10 cc. of the three-ion media was added. Except for a decrease in motility the behavior of larvae in these media was essentially the same as in these solutions without dye. Neutral red, although a powerful accelerator of metamorphosis, was ineffective in bringing about this phase of the life cycle of the larvae. In fact, there have been no cases encountered so far in which neutral red has effected metamorphosis in solutions that inhibit the process. (This is true also of a pH below 6.0.) The only significant differences in the K-free, Ca-free and Na-free media were: (1) In K-free solutions minute amounts of adhesive material were lost and as the ciliated covering was shed, it became entrapped in this fluid; this did not occur in Ca-free and Na-free solutions, for in the former the ciliated cells were shed into the medium and in Na-free there was no noticeable loss of ciliated covering. (2) Larvae first placed in the ion-free media and then removed to sea water within an hour showed obvious elongation only in the case of Na-free media; there was a very slight elongation of larvae placed in K-free media, but the organisms were only one-fourth normal size (probably because of the loss of adhesive fluid). Larvae in these media failed to show the violent photonegative response of the controls (the same concentration of dye and sea water), generally being quite indifferent to light; in general, the organisms behaved remarkably like those in sea water containing an excess of magnesium chloride. The absence of any one of the ions, except perhaps Na, definitely inhibited metamorphosis, probably by accentuating the anaesthetizing effects of magnesium.

Larvae in Mg-free media exhibited the most striking effects, for a deficiency of the ion accentuated the accelerating properties of neutral red, metamorphosis taking place within 15–30 minutes. Organisms that remained in this solution had slightly elongated by 24 hours, whereas those removed to sea water developed

normally but at a somewhat retarded rate, having developed polypides only by 38 hours. (Polypides are normally recognizable before 24 hours.) All these larvae attached rigidly to the substrate. In view of the inhibiting effects of magnesium, these results might have been anticipated.

Neutral red in sea water at reduced temperatures. In previous experiments it had been observed that cold sea water inhibited metamorphosis of the larvae of *B. neritina* (Lynch, 1947). If neutral red accelerates metamorphosis by activating larval enzymes, a reduction of temperature should reduce the effect. To verify this hypothesis two test solutions were made by adding one drop of neutral red/10 cc. of sea water; the temperature of one of these was maintained at 4° C.

TABLE IV

The effects of concentration of neutral red on the duration of the natatory period

I. Larvae in one drop of 0.1% aqueous soln. of neutral red/10 cc. of sea water				II. Larvae in one drop of 0.1% aqueous soln. of neutral red/30 cc. of sea water			
No. larvae	Number unmetamorphosed in hours			No. larvae	Number unmetamorphosed in hours		
	1	2	3		1	2	3
19	2	2	0	45	19	4	1
27	1	0	0	75	64	6	0
30	2	0	0	91	62	4	1
27	3	0	0	19	13	1	1
25	1	0	0	53	39	25	21
45	9	0	0	42	20	6	1
84	9	9	0	298	188	43	4
65	19	6	0	303	243	19	1
42	13	2	0	42	14	8	1
85	1	0	0	30	13	10	3
52	0	0	0	27	14	10	3
22	7	0	0	16	15	8	7
23	3	0	0				
547	70 = 12.8%			1041	704 = 67.2%		

and the other was kept at 12° C. The motility of larvae immersed in these media was greatly reduced by half an hour and the organisms all settled to the bottom exhibiting neither positive nor negative reactions to light. Metamorphosis did not occur at the usual time for larvae in this concentration of neutral red, for only a few had begun the process by an hour and fifteen minutes and these did not attach rigidly as they did at room temperature. The second stender dish (12° C.) was allowed to warm at this time, and by an hour and forty-five minutes only 20% had metamorphosed; these had their internal sacs everted upwards. By four hours only 50% had metamorphosed and these organisms showed no elongation by 24 hours; the controls (larvae in the same concentration of neutral red and sea water at room temperature) had metamorphosed by an hour. Cooling the medium, therefore, inhibited metamorphosis even after the solution came to room temperature.

Neutral red in isotonic solutions. These media consisted of one drop of neutral red/10 cc. of isotonic solutions of the four chlorides. Metamorphosis was not observed in any of them and motility was greatly reduced, never enduring for more than half an hour. Calcium chloride had the most deleterious effect, causing the cilia to dissolve and bringing about cytolysis of the larvae to a marked degree. In the potassium solution ciliated tissue was shed just as it was in sea water containing an excess of this ion (Lynch, 1949a). In the sodium chloride solution the larval covering also disintegrated instead of being pushed inside by tissue from the pallial furrow which had begun to migrate towards the basal end. Magnesium chloride merely anaesthetized the larvae causing the cilia to stand out like a circlet surrounding them. These results were similar to those obtained by using isotonic solutions without neutral red. It is interesting to note that Bradway (1936) had found isotonic calcium chloride to be the most deleterious of all the chlorides in its effect on ascidian larvae; magnesium chloride was relatively non-toxic and inhibited metamorphosis.

Methylene blue in sea water. Methylene blue, though much less effective in accelerating metamorphosis than neutral red, gave results similar to those just described. Only two concentrations were used, one drop of 0.1% aqueous solution/10 cc. and 50 cc. of sea water. The pH was 7.6 and the F.P.D. was 1.71. Motility was considerably greater than in similar concentrations of neutral red and the photo-negative response not only occurred later but was also less pronounced. Although the blue dye was not absorbed as readily nor as extensively as neutral red, the staining reactions were similar, the apical end being almost colorless; whether this was caused by a failure of this region to absorb the dye or by a reduction process is not known. More larvae were geo-negative in the methylene blue media than in those containing neutral red. Except for larvae transferred to sea water after metamorphosis, zooid formation was rather poor in the more concentrated solution; those that attached to the surface, however, developed normally even when they were not transferred to sea water, but geo-negative settings did not.

Discussion

Several explanations of the *modus operandi* of metamorphosis have been offered in the past by various investigators. Thirty years ago Huxley (1922) suggested that unfavorable environmental conditions can bring about the changes, generally cataclysmic, that reduce a free-swimming larva to a state of morphological chaos followed by a constructive phase of differentiation and growth. After comparing similarities between normal metamorphosis in Echinus plutei and dedifferentiation in hydroids and in the ascidians, *Clavelina* and *Perophora*, he concluded that the two processes are essentially similar and predicted that toxic agents would induce precocious metamorphosis in echinoderm larvae.

Certainly copper, $HgCl_2$, $ZnSO_4$, distilled water, isotonic salt solutions, starvation and vital dyes would seem to affect larvae adversely. (For the relative toxicities of neutral red, methylene blue and Janus green, see Child and Rulon, 1936.) Although there is much logic in the proposition of Huxley (1928) that Echinus larvae metamorphose when their increasing weight causes them to sink to the bottom away from the more favorable conditions of food and oxygen at the surface (and this idea would be applicable to ascidians, which become geo-positive

just before setting), the same explanation cannot be used for bryozoan larvae that frequently attach to the surface. Although the internal environment might become unfavorable because of the accumulation of toxic products of metabolism or starvation (since an alimentary canal is absent), the external environment is apparently unchanged just before setting. The hypothesis that an unfavorable environment causes metamorphosis is an unsatisfying one from a chemical viewpoint, since it does not explain why metamorphosis occurs under adverse conditions.

At first sight the hypothesis that larval enzymes are involved in metamorphosis seems to have much in its favor. The observations already presented, as well as those of Bradway (1936) and Zhinkin (1938), on the effects of vital dyes in accelerating metamorphosis would seem to indicate that a stimulation of proteolytic enzymes is somehow involved. On the other hand, Glaser and Anslow (1949) proposed as a tentative hypothesis that copper inactivation of larval enzymes is the "key to morphological disintegration" characteristic of the disruptive phase of ascidian metamorphosis. How, then, can two viewpoints, one of stimulation of larval enzymes and the other of inactivation, be reconciled? It may be that some agents, such as copper, can inactivate certain enzymes, perhaps succinic-dehydrogenase systems, that are necessary for larval life; others may stimulate autolysis. The same net result—larval dedifferentiation—could presumably be attained by stimulating proteolytic enzymes or by inactivating larval oxidation-reduction systems. The failure of bryozoan larvae to develop in copper solutions capable of accelerating metamorphosis (Lynch, 1949a) should be expected, according to the enzyme hypothesis, for copper would apparently be disastrous not only to the larvae but to the development of adult structures as well.

The hypothesis that autolysis initiates metamorphosis and that this process is accelerated by vital dyes leaves much to be desired. Huxley (1922) could find no evidence for believing that cytolyzing enzymes are a necessary hypothesis for explaining the resorptive dedifferentiation that occurs in hydroids and in *Perophora*. Grave (1935) likewise could find no support for Berrill's theory (1929) that phagocytosis of the tail of ascidian tadpoles is the initiator of metamorphosis. If factors causing the acceleration of bryozoan metamorphosis are listed opposite those bringing about retardation, it seems possible that the "colloidal theory of calcium release" may explain most of the facts. Accelerating agents may conceivably effect metamorphosis by a direct action on protoplasmic viscosity.

Accelerators of metamorphosis:	Inhibitors of metamorphosis:
Heat (Marcus, 1926; Lynch, 1949b)	Cold (Lynch, 1947)
Hypertonic solutions (Lynch, 1949a)	Hypotonic solutions (Lynch, 1947)
Absence of magnesium	Excess of magnesium (Lynch, 1949a)
Moderate amount of diffuse light (Grave, 1930)	Absence of light (Lynch, 1949b)
Copper (Lynch, 1949a)	Excess of potassium (Lynch, 1949a)
Large excess of calcium	Absence of Na, K or Ca in three-ion combinations
Vital dyes	A pH below 6.0 (Lynch, 1949a)

It seems more than merely coincidental that inhibitors of metamorphosis, such as cold sea water, an excess of magnesium or potassium, are also agents that have an anaesthetizing effect. Interpreted in terms of the calcium-release theory, in-

hibition of metamorphosis may be merely a kind of anaesthesia, reducing the activity of larvae to a minimum and thus forestalling the unfavorable effects of starvation. (For the narcotizing effects of magnesium and potassium see Heilbrunn, 1943, p. 520.) The absence of calcium, at least, should accentuate the anaesthetizing effects of magnesium (Heilbrunn, 1943, p. 531); what effects the absence of sodium or potassium have on protoplasmic viscosity are not well known. It seems logical to attribute the inhibitory effects of hypotonic sea water on the larvae of *B. neritina* to a decreased protoplasmic viscosity caused by absorption of water. (If, however, sea water is diluted by 50% with distilled water and larvae are placed in it for ten minutes and then returned to sea water, metamorphosis and zooid formation take place.) Accelerating agents, on the other hand, rather closely parallel those that stimulate protoplasm or cause a clotting of the interior of cells. Thus, hypertonic solutions, which accelerate metamorphosis, are known to cause calcium release from the cortical layer resulting in a concomitant increase of the viscosity of the inner protoplasm. Since light can also release calcium and increase the viscosity of the interior protoplasm (Alsup, 1942), an absence of light may presumably have the opposite effect—hence the inhibiting effects of darkness. (It is interesting to note that Knight-Jones, 1951, observed that the trochophores of *Spirorbis* behave like the larvae of *Bugula* in darkness; both attach to the surface film predominantly. In fact, the pre-fixational activities of *Spirorbis* are so remarkably similar to those of *Bugula* that the parallel suggests that behavioral patterns may be preserved through evolutionary changes.) The action of vital dyes may be interpreted, perhaps, as direct effect on protoplasmic viscosity rather than an indirect one by activating proteolytic enzymes. The studies of Alsup (1941, 1942) on the photodynamic action of rose bengal and eosin on protoplasmic viscosity of the interior of cells, increasing it in the presence (but not in the absence) of light, suggest that neutral red and methylene blue may have effects similar to the dyes he used. All these dyes seem to affect protoplasm in much the same way. The writer found eosin effective in initiating metamorphosis, but it was even less potent than methylene blue, and Hassett (1941) found that rose bengal, eosin, neutral red and methylene blue all had similar effects on the light responses of *Peranema tricophorum*. Whether vital dyes affect the protoplasm directly or only indirectly via an enzyme system is problematical, for Heilbrunn (1943, p. 538) stated that "the clotting of protoplasm may well be related to some proteolytic enzyme action." At present it is generally believed that dyes with a photodynamic action affect exposed —SH groups of the protein molecule. (*Cf.*, Calcutt, 1951.) The observations of Angerer (1937, 1942) that copper chloride (an accelerator of metamorphosis) increases the viscosity of sea urchin eggs and of the protoplasm of *Amoeba dubia* may indicate that copper also has a direct effect on larvae rather than an indirect one by inactivating larval enzymes. At any rate, the hypothesis of Glaser and Anslow (1949), emphasizing the role of copper in inactivating larval enzymes, seems to be inadequate, since it fails to take into consideration the host of other substances that accelerate metamorphosis.

There is, therefore, some cumulative indirect evidence for the hypothesis that anaesthetic agents inhibit metamorphosis and that factors causing a release of calcium accelerate it; if it is a tenable one, further experiments should show a general effect of all narcotizing agents. (It is interesting to note that Grave

(1935) observed a narcotizing effect of something in the medium when adrenalin was used in experiments on ascidian metamorphosis; attachment was inhibited.) If coagulating agents can induce metamorphosis, thromboplastic-like substances should hasten the process and heparin should inhibit it. Harding (1951) found that injury substance from minced frog muscle initiated cell division parthenogenetically in *Arbacia* eggs. This substance, according to Heilbrunn *et al.* (1936), is apparently a thrombin-like material. Heilbrunn and Wilson (1949, 1950) found that heparin, a bacterial polysaccharide acting like heparin and dicumarol, inhibited cell division in the eggs of *Chaetopterus*. None of these substances has been tried for inhibiting effects on bryozoan larvae, but injury substance made from frog muscle according to the procedure outlined by Harding (1951) not only failed to accelerate metamorphosis but actually inhibited it. Seven or eight trials with this substance, extracted from frog muscle on four or five occasions, all gave negative results. At a pH of 4.5, found to be effective by Harding (1951), the larvae became immobilized immediately and merely disintegrated after a few hours. Since metamorphosis is inhibited at a pH as low as 4.5, injury substance was tried within a pH range of 5.7, 7.6 and 8.3. (KOH and borate buffers were used in raising the pH of the originally acid injury substance) in combinations ranging from 3 cc. injury substance/7 cc. of sea water, the reverse proportions and other mixtures. In all cases, from pH 5.7–8.3 the results were similar. The larvae gradually lost adhesive material from the internal sac, became stuck to one another, eventually disentangled themselves and continued to swim for 8–10 hours. Metamorphosis could not be induced even when neutral red was subsequently added to these media after five hours exposure. Results similar to these were obtained with the whole blood of frogs added to sea water. In some of these experiments with thrombin-like substances the internal sac ruptured completely without being everted; but the process was mechanical and not one in which the larvae actively participated. In all cases the entire medium in which larvae were placed became extremely cloudy because of congealed adhesive material. Yet the fact that these substances did cause emission of attachment fluid might lead to the supposition that clotting agents in exactly the right proportions might prove effective in accelerating metamorphosis. The theory of calcium release would seem to offer some explanation for the accelerating effects on ascidian larvae caused by an extract of muscle tissue of rabbits killed by x-rays (Bertholf and Mast, 1944) and by whole thyroid gland (but not thyroxine), mantle tissue, etc. (Grave, 1935). In fact, Grave stated (1935, p. 288) that, "If muscle tissue of whatever origin should prove to contain an accelerating substance, an explanation might be afforded for the exceptional results of experiment 191, in which an extract of mantle and foot tissue of a snail . . . induced 100% metamorphosis in a group of larvae of *Polyandrocarpa*. . . ." Although Grave considered that the effective agent in muscle might be an endoenzyme or ferment, it seems more likely, in view of the recent experiments of Heilbrunn *et al.* (1946), that a thromboplastic-like substance may be involved. Like carcinogens and like parthenogenetic agents, factors influencing metamorphosis are many and varied, apparently having little or no relation to one another. Only extensive experiments in the future can bring the complicated process of metamorphosis nearer to an acceptable solution.

The writer expresses his gratitude to Professors H. B. Steinbach and H. W. Stunkard for reading this paper and for making many valuable suggestions for its revision. Dr. Stunkard made these experiments possible by generously providing space in his laboratory.

Summary

1. In calcium-free van't Hoff's solution and both Na-free and K-free mixtures having the other ions in the same proportion as in sea water at a pH of 7.1–8.0, the larvae of *Bugula flabellata* behaved exactly like those in sea water containing an excess of $MgCl_2$. Phototropic reactions were lost, ciliated tissue was shed as material migrated basally from the pallial furrow and metamorphosis did not occur.

2. In isotonic Mg-free solutions (pH = 8.0) metamorphosis was greatly accelerated, fixation occurring within 30 minutes to 3 hours in the majority of larvae; these organisms developed well-formed zooids in 10 hours. (Normally only about 77% of the larvae metamorphose by 12 hours.)

3. Neutral red, methylene blue and, to a lesser extent, eosin accelerated metamorphosis. Neutral red, the most potent of the three, was effective in concentrations of 1 : 1,000,000 parts of sea water; methylene blue caused acceleration in concentrations of 1 : 500,000 parts. At a concentration of 1 : 100,000 parts of neutral red the average duration of the natatory period was about an hour.

4. Metamorphosis failed to occur in Ca-free, Na-free and K-free media to which neutral red had been added in proportions of 1 : 100,000 parts. The same concentration of neutral red in isotonic Mg-free media (pH = 8.0) had a greater accelerating effect than when the dye was added to sea water in the same proportions. A reduction of temperature to 4° C. to 12° C. of sea water containing neutral red in parts of 1 : 100,000 antagonized the accelerating effects of neutral red. The staining reactions of neutral red and the problems involved in the study of metamorphosis are also discussed.

LITERATURE CITED

Alsup, F. W., 1941. Photodynamic studies on Arbacia eggs. *Biol. Bull.*, **81**: 297.

Alsup, F. W., 1942. The effects of light alone and photodynamic action on the relative viscosity of Amoeba protoplasm. *Physiol. Zool.*, **15**: 168–184.

Angerer, C. A., 1937. The effects of salts of the heavy metals on protoplasma I. The action of cupric chloride on sea-urchin eggs. *J. Cell. Comp. Physiol.*, **10**: 183–197.

Angerer, C. A., 1942. The action of cupric chloride on the protoplasmic viscosity of *Amoeba dubia*. *Physiol. Zool.*, **15**: 436–442.

Armstrong, E. G., and L. M. Miall, 1945. Raw materials from the sea. XI. Constructive Publications Ltd. Leicester, England. Pp. 1–164.

Berrill, N. J., 1929. Studies in tunicate development. Part I: General physiology of development of simple ascidians. *Phil. Trans. Royal Soc. London, Ser. B*, **218**: 37–78.

Bertholf, L. M., 1945. Accelerating metamorphosis in the tunicate, *Styela partita*. *Biol. Bull.*, **89**: 184–185.

Bertholf, L. M., and S. O. Mast, 1944. Metamorphosis in the larva of the tunicate, *Styela partita*. *Biol. Bull.*, **87**: 166.

Bradway, Winnefred, 1936. The experimental alteration of the rate of metamorphosis in the tunicate, *Clavelina huntsmani* (Van Name). *J. Exp. Zool.*, **72**: 213–225.

Calcutt, G., 1951. The role of radiation in photodynamic action. *J. Exp. Biol.*, **28**: 537–541.

Child, C. M., and Olin Rulon, 1936. Differential reduction of vital dyes in certain Oligochetes. *J. Exp. Zool.*, **74**: 427–449.

CORRÊA, D. D., 1948. A embriologia de *Bugula flabellata* (J. V. Thompson). *Bol. Fac. Fil. Ciênc. e Letr. Univ. São Paulo. (Zool).*, **13**: 7–71.

GLASER, OTTO, AND GLADYS A. ANSLOW, 1949. Copper and ascidian metamorphosis. *J. Exp. Zool.*, **111**: 117–140.

GRAVE, B. H., 1930. The natural history of *Bugula flabellata* at Woods Hole, Massachusetts, including the behavior and attachment of the larva. *J. Morph.*, **49**: 355–383.

GRAVE, CASWELL, 1935. Metamorphosis of ascidian larvae. Papers from the Tortugas Lab. Reprinted from Carnegie Inst. of Wash. Pub. No. 452: 209–292.

GRAVE, CASWELL, 1941. Further studies of metamorphosis of ascidian larvae. *Biol. Bull.*, **81**: 286–287.

GRAVE, CASWELL, 1944. The larva of *Styela (Cynthia) partita:* Structure, activities and duration of life. *J. Morph.*, **75**: 173–191.

GRAVE, CASWELL, AND P. A. NICOLL, 1936. Accelerators of metamorphosis of larvae of *Phallusia nigra* and species of *Polyandrocarpa.* Ann. Rept. Tortugas Lab., Carneg. Inst. of Wash., 1935–1936: 84–85.

HARDING, DRUSILLA, 1951. Initiation of cell division in the Arbacia egg by injury substances. *Physiol. Zool.*, **24**: 54–70.

HASSETT, C. C., 1941. The effect of dyes on the response to light in *Peranema trichophorum. Biol. Bull.*, **81**: 285.

HEILBRUNN, L. V., 1943. An outline of general physiology. Second Edit. W. B. Saunders Co., Philadelphia.

HEILBRUNN, L. V., D. L. HARRIS, P. G. LE FEVRE, W. L. WILSON AND A. A. WOODWARD, 1946. Heat death, heat injury and toxic factor. *Physiol. Zool.*, **19**: 404–435.

HEILBRUNN, L. V., AND W. L. WILSON, 1949. The effects of heparin on cell division. *Proc. Soc. Exp. Biol. Med.*, **70**: 179–182.

HEILBRUNN, L. V., AND W. L. WILSON, 1950. The prevention of cell division by anti-clotting agents. *Protoplasma,* **39**: 389–399.

HUXLEY, J. S., 1922. Dedifferentiation in Echinus larvae, and its relation to metamorphosis. *Biol. Bull.*, **43**: 210–239.

HUXLEY, J. S., 1928. Experimentally-induced metamorphosis in Echinus. *Amer. Nat.*, **62**: 363–377.

KNIGHT-JONES, E. W., 1951. Gregariousness and some other aspects of the setting behavior of Spirorbis. *J. Mar. Biol. Assoc.*, **30**: 201–222.

KOEHRING, VERA, 1930. The neutral red reaction. *J. Morph. and Physiol.*, **49**: 45–138.

KOEHRING, VERA, 1931. Thermal relationships in the neutral red reaction. *J. Morph.*, **52**: 165–195.

LEFEVRE, P. G., 1948. Further chemical aspects of the sensitization and activation reactions of Nereis eggs. *Biol. Bull.*, **95**: 433–446.

LYNCH, W. F., 1947. The behavior and metamorphosis of the larva of *Bugula neritina* (Linnaeus): Experimental modification of the length of the free-swimming period and the responses of the larvae to light and gravity. *Biol. Bull.*, **92**: 115–150.

LYNCH, W. F., 1949a. Acceleration and retardation of the onset of metamorphosis in two species of Bugula from the Woods Hole region. *J. Exp. Zool.*, **111**: 27–55.

LYNCH, W. F., 1949b. Modification of the responses of two species of Bugula larvae from Woods Hole to light and gravity: ecological aspects of the behavior of Bugula larvae. *Biol. Bull.*, **97**: 302–310.

MARCUS, ERNST, 1926. Beobachtungen und Versuche an levenden Süsswasserbryozoen. *Zool. Jahrb. Syst.*, **52**: 279–351.

NEEDHAM, JOSEPH, 1950. Biochemistry and morphogenesis. Cambridge University Press.

PRYTHERCH, H. F., 1934. The role of copper in the setting, metamorphosis and distribution of the American oyster, *Ostrea virginica. Ecol. Monog.*, **4**: 47–107.

RUNNSTRÖM, J., 1917. Analytisch Studien über die Seeigelentwicklung. III. *Arch. f. Entw.*, **53**: 223–329.

WEISS, PAUL, 1928. Experimentelle Untersuchungen über die Metamorphose der Ascidien. *Biol. Zentr.*, **48**: 69–79.

ZHINKIN, L. N., 1938. Stimulation of metamorphosis in ascidian larvae. *C. R.* (Doklady) *Acad. Sci. U.S.S.R.*, **18**: 213–216.

THE FINE STRUCTURE OF THE PERITROPHIC MEMBRANES OF CERTAIN INSECTS

E. H. MERCER[1] AND M. F. DAY

Chemical Physics Section, Division of Industrial Chemistry, C. S. I. R. O., Melbourne, Victoria, and Division of Entomology, C. S. I. R. O., Canberra, A. C. T., Australia

The structure and formation of the peritrophic membrane (p.m.) of many insects have been studied extensively (Wigglesworth, 1950; Richards, 1951). Although the need for more detailed knowledge of the fine structure of the membrane was apparent, the results of early attempts to use electron microscopy were not encouraging (Richards and Korda, 1948). Later, Huber and Haasser (1950) reported some details of the p.m. of *Dixippus;* they found it to be a (p. 397) "more or less regular network, probably fibrous, with a thin film stretched across the holes of the network." Shortly afterwards, networks with meshes approximately 0.2 μ across and composed of fine fibrils were found electron microscopically by Lagermalm, Philip and Gralén (1950) in the excreta of the clothes moth larvae (*Tineola bisselliella*). These networks originated from the insect, and not from the wool which formed its food; it was not proved that they originated from the peritrophic membrane, although this seemed likely because of the similarity between these networks and those described by Huber and Haasser. Huber (1950) reported further details of the structure of the p.m. in *Periplaneta orientalis, Tenebrio molitor* and *Bombyx mori.* In all except the last the characteristic networks were found.

Cross-sections of the peritrophic membrane examined in the light microscope show it to consist of several loosely adhering layers, each roughly 0.5 μ to 1 μ thick. Since the networks are much thinner than this, it appears that each thicker layer can be further separated into thinner layers.

There are said to be two methods of formation of peritrophic membranes. In one, the membrane originates solely from specialized cells at the anterior end of the midgut and in the other the membrane is said to be produced by delamination of a series of concentric lamellae from the surface of all the cells of the midgut. The first type is said to occur in Diptera and Dermaptera and the second in certain Orthoptera, and in hymenopterous and coleopterous larvae (Wigglesworth, 1950). There is, however, still much uncertainty about the origin of the membranes and the nature of the secretory processes producing them. It is possible that a study of the fine structure will help to clarify this problem and to throw light on the physiology of absorption of food and passage of enzymes.

The present investigation began with the chance discovery in saliva of *Periplaneta* of membranes similar to those described by Huber and Haasser and by Lagermalm, Philip and Gralén. This present paper deals with the fine structure of these membranes and those of other insects, and the problem of the formation of the membranes.

[1] Present address (until February 1, 1953): Textile Research Institute, Princeton, New Jersey.

Experimental

A. Sources of material

Most of the material was obtained from the cockroach *Periplaneta americana* (L.) which proved such a satisfactory source that all the illustrations are derived from this insect. However, it is desirable in the first place to determine whether conclusions drawn from a study of this species have a general application; and secondly, since two types of p.m. corresponding to two methods of formation have been described, to examine and compare membranes of these two alleged types. This survey has not yet been carried very far, but the p.ms. of two other species have been found to contain membranes of the same general construction as those of *Periplaneta;* these are *Locusta migratoria* (L.) and *Galleria mellonella* (L.). In the following species membranes were not discovered: the dermapteran, *Titanolabis colossea* (Dohrn); the larva of the blowfly, *Lucilia cuprina* (Wied.); larva of the cabbage white butterfly, *Pieris rapae* (L.), of the dermestid beetle *Attagenus piceus* (Oliv.), and the worker honeybee, *Apis mellifera* (L.). A negative finding, it is emphasized, must be regarded with reserve since an element of chance enters into a search of this nature, and a more extensive search may lead to a positive result. It is not, therefore, possible to state with certainty yet whether two varieties of membrane exist.

Because networks had been previously found in *Tineola* excreta (Lagermalm, Philip and Gralén, 1950), several examinations were made of aqueous suspensions of this material without finding other than traces of fibrils.

B. Preparation of material for electron microscopy

Two procedures were suitable for the preparation of material from *Periplaneta* for examination in the electron microscope: (1) stimulation of the insect to cause saliva to exude from the mouth; and (2) dissection of the peritrophic membrane followed by its disintegration in water.

1. Saliva. The oral exudate from *Periplaneta* consists of a clear fluid which was dried directly on the ordinary specimen screen. The dried screen was washed several times with water in order to remove soluble salts, etc. This technique gave quite clean fields and about one quarter of all screens so prepared yielded membranes.

Following the discovery of membranes in *Periplaneta* possible sites of origin were considered, although their similarity to the structures described by Huber and Haasser (1950) made the peritrophic membrane seem the most probable. Proteinase activity could not be detected in the saliva as might have been expected if the contents of the midgut, which are certainly regurgitated into the crop (Day and Powning, 1949), had been able to reach the mouth parts. The possibility, therefore, that the chitinous intima of the salivary reservoirs or crop might be the source of the membranes was explored. Membranes were searched for in crop scrapings and in the contents of the salivary reservoirs, without success. However, when p.ms. were dissected and treated according to the method described below, abundant membranes were immediately discovered. These were far more extensive than the fragments found in saliva and more details of their structure were apparent. It seems certain that the networks of the saliva were in fact fragments

of the p.m., thus indicating regurgitation of midgut contents and suggesting that the inability to detect proteinase activity in the saliva was due to deficiencies of the technique.

2. Dissection. Dissection of the p.m. proved the most reliable source of material. The membrane and contents were dissected from the midgut and allowed to stand in water at about 1–2° C. from a few hours to several days with occasional shaking. Finally, vigorous shaking dispersed the membrane into fragments. After the coarser fragments had settled, drops of the permanent suspension were dried on the specimen screens.

The material obtained from all of these methods of preparation has been subjected to the action of the digestive enzymes of the insect and this should be kept in mind when considering the micrographs.

C. Electron microscopy

The microscope used was an R.C.A. Type EMU, and standard methods of specimen mounting, shadowing and examination were employed. Most screens were shadowed with uranium before examination to facilitate discovery of the p.m. fragments and all the plates reproduced here illustrate such specimens. However, the general structural features of the membranes were visible without shadowing. Attempted staining with phosphomolybdic acid did not result in any differentiation of structure.

Results

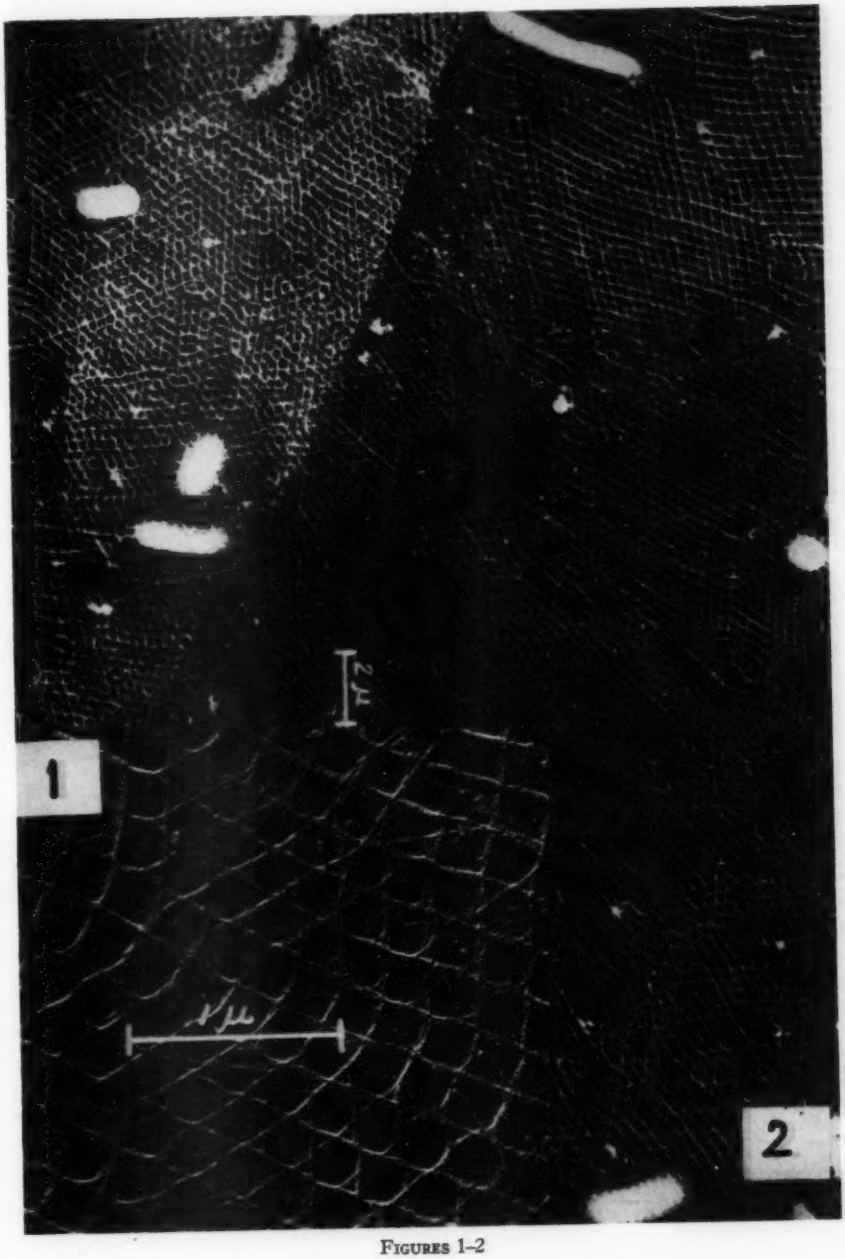
Occasional thick sheets were noted in aqueous suspensions of dissected p.ms. These were too thick for examination with 50 K.V. electrons. It was assumed that such material represented the total p.m., *i.e.*, the layers visible as separate sheets in the light microscope, not resolved into components. At the edges of such sheets signs of fraying into thinner membranes and fibrils were sometimes seen.

In preparations derived from saliva or directly from the dissected membrane, the most commonly occurring and easily recognized constituent is the *"regular fibrillar network"* (Figs. 1 and 2), as this structure will be referred to in this paper. Other apparently different kinds of membranes were also found, although these may really be variants or defective forms of the regular networks. For example, a honeycomb-like formation (*"honeycomb network"*) was occasionally observed (Fig. 3), and frequently superimposed upon the regular networks there occurred a distinct layer of irregularly arranged fibrils (*"irregular meshwork"*) (Fig. 6). Adhering to most membranes and often interpenetrating the fibrillar components was found an amorphous ground substance which, since it seems to be partly removed by the procedures associated with preparation, may consist of a less resistant substance than the fibrils. The electron micrographs alone do not afford

---

Figure 1. Fibrillar membrane from *Periplaneta*. Note areas with approximation to hexagonal symmetry and various types of defective structures. Each strand of the meshwork consists of several fine fibrils. Magnification: 32,000 ×.

Figure 2. Extensive area of the fibrillar membrane. Notice folds and structural irregularities. The size of the mesh may be compared directly with the bacteria also present. Magnification: 5200 ×.



FIGURES 1–2

definite proof that this amorphous substance is a structural constituent rather than a contaminant. However, since it seems to assist in maintaining the coherence of the total formation and is so thoroughly imbedded in the irregular meshwork, it will be assumed that it is a genuine component.

The regular fibrillar networks

The characteristics of these membranes can be appreciated from a study of Figures 1, 2 and 4. They are built from long straight strands or groups of fibrils of diameter probably less than 100 Å and of indefinite length. Ideally the structure tends towards hexagonal symmetry which can be seen in certain portions of the network shown in Figure 1. There are three sets of fibrils placed at 60° and forming triangular or hexagonal meshes (Fig. 8D). Commonly the structure is defective. A set of fibrils may be lacking, resulting in four-sided meshes (Fig. 8E), or the shape may be distorted. The number of separate fibrils composing a strand appears to vary, but is usually about four. Figure 4 illustrates fibrils protruding from the torn edge of a network. The size of the meshes is variable but on the average the parallel strands are about 0.15 μ to 0.2 μ apart. The networks were commonly found stretched flat across the supporting screens with occasional puckers or folds and frequently covered very considerable areas (*e.g.*, greater than 100 $\times$ 200 μ). The individual fibrils themselves seem to be structureless, although occasional ambiguous evidence of periodic nodulation was seen after shadowing.

The component fibrils of the strands are neither twisted together to form the strands nor regularly woven into the meshwork. They seem to form a purely random deposition as would result if a collection of straight fibers were sorted out upon an hexagonal array of pegs.

The irregular meshworks

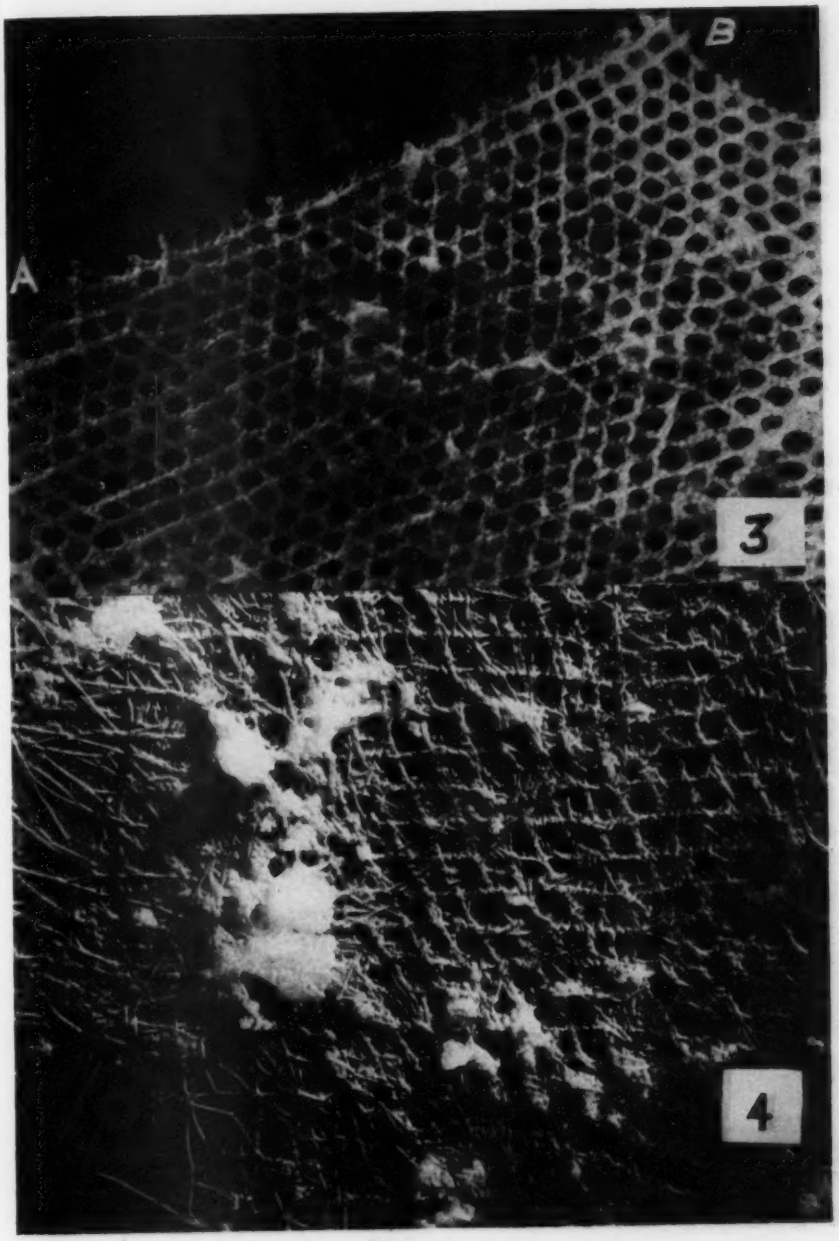
These formations seem to consist of fibrils similar in size to those forming the regular networks, but are less straight. They appear to be bent and felted together and imbedded in the amorphous ground substance which, by interpenetrating adjacent networks, very probably cements the whole laminated structure together. Occasional areas of ground substance lacking fibrils were noted. Some of the fibrils seem to continue directly into those composing the regular networks. This would suggest that the two types of membranes are essentially similar but that the organization is defective in one case.

The honeycomb networks

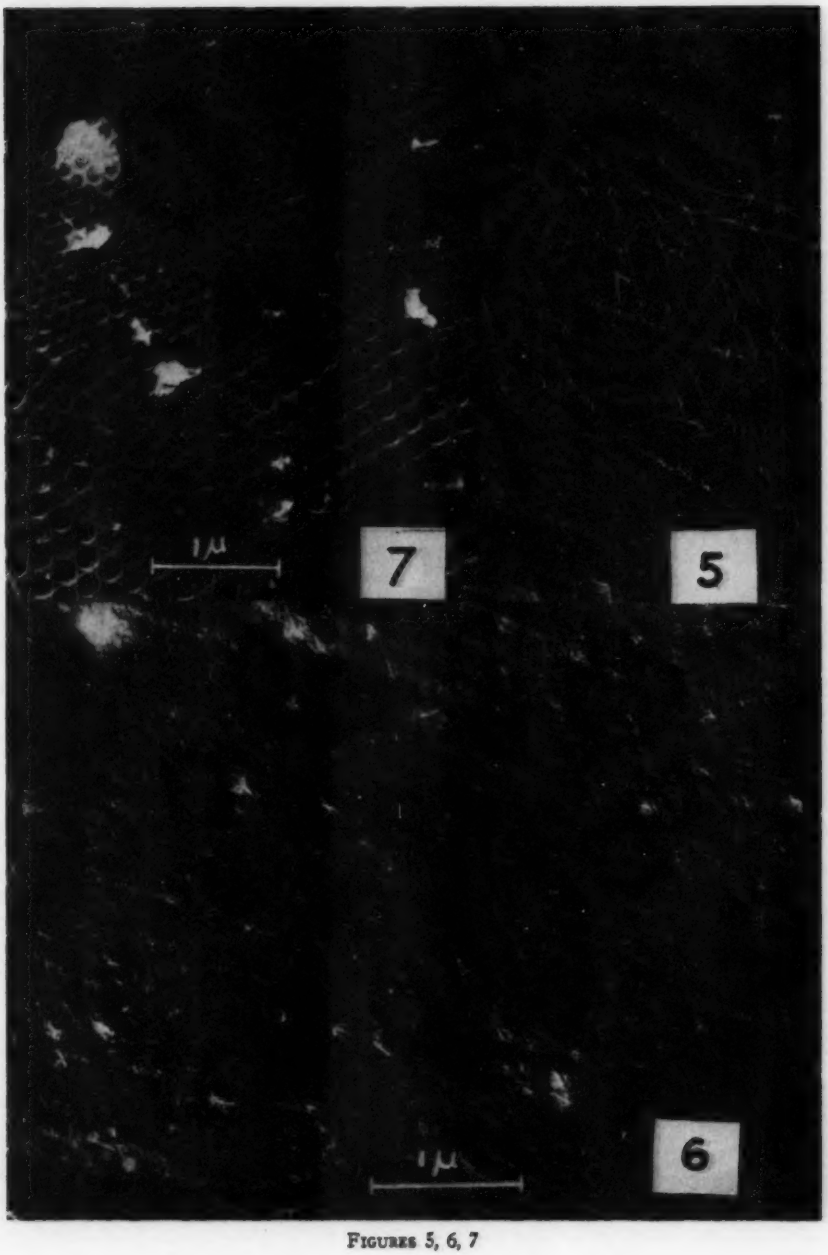
As can be seen from Figure 3 these networks present a distinctive appearance. In particular they tend to fracture along a straight line of holes, presenting a clean break distinct from that shown by the fibrillar networks (Fig. 4). They may, however, be of the same basic construction as the fibrillar network but consist of fewer

FIGURE 3. Example of "honeycomb network." Note straight edge at AB where membrane has broken. Magnification: 22,000 $\times$.

FIGURE 4. Edge of fibrillar network showing fraying into fibrils. Patches of ground substance containing fibrils may be seen. Magnification: 22,500 $\times$.



FIGURES 3–4



FIGURES 5, 6, 7

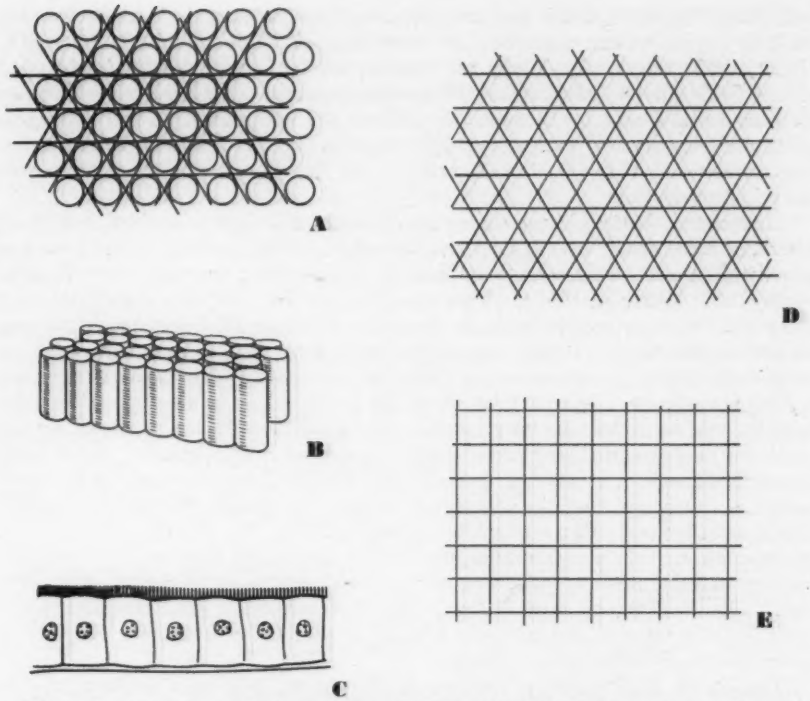


FIGURE 8. (A) Close-packed arrangement of spherical particles illustrating the formation of the hypothetical template surface having hexagonal symmetry. The way in which fibrils are deposited to form a hexagonal network (D) is indicated. (B) Similar surface of close-packed cylinders. (C) Diagram of striated border of midgut cells which may be formed by the aggregation into coarser fibrils of a finer structure such as shown at (B). (D) Regular fibrillar network as deposited on template of type (A). (E) Square network as deposited on a template of particles in square array formed by distortion of a close-packed array.

fibrils heavily encrusted with the amorphous ground substance. The holes may be either larger or smaller than the average size of the apertures in the regular networks.

DISCUSSION

A. Structure and function of peritrophic membrane

The coaxial lamellae composing the p.m. appear to possess a complex fine structure. Each layer seems to consist of a fine regular network associated with less

FIGURE 5. Example of imperfectly developed network. Magnification: 22,500 ×.

FIGURE 6. Fibrillar network with the irregularly ordered meshwork of fine fibrils partly superimposed. Notice presence of amorphous ground substance. Magnification: 22,500 ×.

FIGURE 7. A fibrillar network with a honeycomb structure lying above it. Magnification: 19,000 ×.

well defined layers of fibrils and amorphous ground substance. The fibrillar network is obviously that described by other authors (Huber and Haasser, 1950; Huber, 1950; Lagermalm, Philip and Gralén, 1950). The irregular meshwork is always found directly adhering to the fibrillar network, as in Figure 6. Here and there an actual continuity between the fibrils of the irregular mesh and the regular hexagonal network can be traced, suggesting that these two structures are formed from similar materials (fibrils and cement), but that in one case the regular order has failed to develop.

Histochemical studies have shown that both chitin and protein occur in the p.m. Although some doubt may exist as to the origin of the protein, it has been suggested that there is a chitinous framework with protein incorporated (Wigglesworth, 1950; Richards, 1951). This conception of the structure of the total membrane is in good agreement with the details now revealed by electron microscopy. It is most desirable to identify chemically the several components recognized, but unfortunately little in the nature of electron-histochemistry has been attempted up to the present time. In particular, since the average atomic weights of the atoms in chitin and in protein are of a similar order, a differentiation of these two materials on the basis of their electron-scattering power is not possible. Since chitin is usually fibrous, it seems not unlikely that the fibrillar component of the membranes is chitin and that the amorphous matrix or ground substance is protein. This conclusion was also reached by Huber (1950). In accord with the usual role of chitin as a skeletal substance, the fibrillar component may serve a mechanical function as reinforcement providing strength and coherence to the lamellar structure. The ideal arrangement of the fibrils, *i.e.*, three sets of parallel strands placed at 60° to each other, is mechanically adapted to the task of forming a tough membrane not readily torn in any direction.

The protein constituents of the complex, assuming these are represented by the continuous ground substance, might be expected to be the determining factor in the transport of solutes across the membrane. The networks, assuming these are more or less continuous, could of course act as coarser filters and prevent the passage of organisms, which might be able to digest and penetrate the protein component alone. The relative sizes of mesh aperture and bacteria may be seen in Figure 2, although the bacteria apparently entrapped here probably result from contamination occurring subsequent to dissection.

The broad features of membrane structure revealed in the p.m. may possibly prove to be of general occurrence, since the complex, fibrous chitin plus protein, is also found in insect cuticles (Richards, 1951) and closer examination of these may reveal fine fibrils of chitin embedded in an amorphous ground substance of a protein nature. Ribi (1951) has, in fact, reported the occurrence of fine chitinous fibrils in arthropod cuticle.

B. The formation of the peritrophic membrane

Certain aspects of the problem of the formation of the p.m. have been studied in sections by means of the light microscope. This work has been summarized by Wigglesworth (1950). Reference has been made above to the two methods of formation which have been suggested. A consideration of the fine structure of the membrane offers further data relevant to this problem. The features which seem to

be of significance are the lamellar structure and the two-dimensional order exhibited by certain of the networks. Both of these features suggest that these particular membranes are examples of exfoliation. In the first place a laminated structure can be readily imagined to form by the shedding of successive sheets from a secreting surface; and secondly, the fine structure of the individual networks could be plausibly explained if we suppose that the fibrils were laid down on a "template" possessing hexagonal symmetry.

It is worthwhile to speculate concerning the essential features of such a template. If the midgut cell surface possesses a pattern having hexagonal symmetry, the simplest method of achieving this is by the close packing of spheres or of circular rodlets. The outer surface could, for instance, be pictured as a close-packed array of spherical corpuscles (Fig. 8A) or of packed rodlets (Fig. 8B). The idea of packed rodlets is attractive because such a formation by aggregating to a coarser structure on fixation would simulate the striated border (diagrammed in Figure 8C) seen to fringe the midgut cells of most insects when viewed in the light microscope. It could then be assumed that the secretion, from which the fibrils separate spontaneously, deposits these fibrils directly in the "grooves" of the patterned surface (Figs. 8A and 8B). Those immediately adjacent to the patterned surface would be formed into the regular network; the excess of fibrils in the secretion more distant from the template surface would be less well ordered and form the mass of irregular fibrils embedded in the amorphous matrix which is found adhering to the regular networks. The matrix in which these fibrils are embedded is probably a second substance present in the secretion which has not the property of forming fibrils. In this way an intimately mixed chitin and protein (?) complex may result. The production of lamellae of structure alternating between a fibrillar texture and an amorphous texture would ultimately depend upon an alternating synthetic activity of the secreting cells which would determine the ratio of the precursors of these components in the secretion.

Since the networks extend over an area greater than that of a single midgut epithelial cell, the hypothetical hexagonal template must preserve the same orientation in adjacent cells. Picken, Pryor and Swann (1947), discussing the similar problem of orientation in cuticles, believe such a coordination unlikely and so are opposed to relating orientation to an underlying organization of the cell surface. However, having regard for the compressed packing of the cells of the columnar epithelium, it seems clear that the hexagonal shape of the cell ends themselves could determine the orientation of the close packing of the particles on their surfaces.

A suggestion similar in principle has been proposed to account for the origin of a two-dimensional array of fibrils observed on the earthworm cuticle by Reed and Rudall (1948) and for the formation of the radiolarian skeleton by Thompson (1942).

The authors are greatly indebted to Mr. J. Farrant for his invaluable suggestions and assistance with the electron microscopy; to Mr. D. F. Waterhouse for providing some of the material; to Mrs. N. Dewsnap for technical assistance; to Mr. L. A. Marshall for the preparation of the text figures; and to many colleagues for helpful discussions.

SUMMARY

1. Some details of the fine structure of the peritrophic membrane of *Periplaneta* and of several other insects have been determined electron microscopically.

2. The membrane appears to be a complex structure of which the most characteristic and resistant component is a fibrillar network. A second component is a layer closely associated with the fibrillar network, consisting of unorganized fibrils embedded in an amorphous ground substance. The layers may be loosely associated and become separated on shaking the dissected membrane in water. The fibrils probably consist of chitin and the ground substance of protein.

3. The fibrillar network generally consists of three systems of parallel fibrillar strands placed at 60° to each other; thus it possesses hexagonal symmetry. A variety of defective arrangements, arising from mesh distortion, suppression of one set of fibrils and other grosser defects, may occur. The diameter of the fine fibrils composing the strands is about 100 Å and there may be several in each strand. The separate strands of a system of fibrils are about 0.15 to 0.2 μ apart. The fibrillar networks are well adapted to the formation of tough sheets, not readily torn.

4. Membranes of this nature were also found in *Locusta migratoria* and *Galleria mellonella*.

5. The nature of these membranes suggests that they are formed by delamination from a surface. This view of the mode of formation may account for the development of the fibrillar arrangement if it is assumed that the fine fibrils are deposited from a secretion onto a surface bearing an hexagonal pattern to act as a template.

LITERATURE CITED

DAY, M. F., AND R. F. POWNING, 1949. A study of the processes of digestion in certain insects. *Aust. J. Sci. Res., Ser. B*, **2**: 175–215.

HUBER, W., 1950. Recherches sur la structure submicroscopique de la membrane peritrophique de l'intestin moyen chez quelques insectes. *Arch. Anat. Histol. Embryol.*, **33**: 1–19.

HUBER, W., AND C. HAASSER, 1950. Electron microscope study of the peritrophic membrane in *Dixippus morosus*. *Nature*, **165**: 397.

LAGERMALM, G., B. PHILIP AND N. GRALÉN, 1950. Occurrence of a network in the excrement from the larva of the clothes moth. *Nature*, **166**: 484.

PICKEN, L. E. R., M. G. M. PRYOR AND M. M. SWANN, 1947. Orientation of fibrils in natural membranes. *Nature*, **159**: 434.

REED, R., AND K. M. RUDALL, 1948. Electron microscope studies on the structure of earthworm cuticles. *Biochim. Biophys. Acta*, **2**: 7–18.

RIBI, E., 1951. Submicroscopic structure of fibers and their formation. *Nature*, **168**: 1082–1083.

RICHARDS, A. G., 1951. The integument of arthropods. Univ. of Minnesota Press, Minneapolis.

RICHARDS, A. G., AND F. H. KORDA, 1948. Studies on arthropod cuticle. II. Electron microscope studies of extracted cuticle. *Biol. Bull.*, **94**: 212–235.

THOMPSON, D'ARCY, 1942. Growth and form. Cambridge University Press. Cambridge, England.

WIGGLESWORTH, V. B., 1950. The principles of insect physiology. 4th Edition. Methuen & Co. Ltd., London.

BLEEDING AND COAGULATION IN SOME BERMUDAN CRUSTACEA [1]

PETER R. MORRISON AND KATHERINE C. MORRISON

Departments of Zoology and Physiology, University of Wisconsin, Madison 6, Wisconsin

The blood of crustaceans has long been of interest to zoologists and many observations on the coagulation function are to be found in the early literature. Glavand (1948) cites a score of studies prior to 1893, two dozen more between this date and 1920 but only a handful thereafter. These studies consider the nature of crustacean coagulation, contrasting it to that in vertebrates, and a number compare various species in regard to intensity and type of mechanism. Tait (1911) recognized three types of animals: first, those with cellular agglutination alone; second, those with cellular agglutination together with some further clotting of the plasma (*i.e.*, with fibrinogen); and third, those with negligible cellular agglutination but substantial clotting of the plasma. The second group, those with both mechanisms, forms a continuous series of intergrades between the first and third groups with either agglutinating cells or fibrinogen. These distinctions were all based on qualitative observations as to the strength and properties of the coagula since no quantitative measurements of the mass of cellular material or of fibrinogen were made. Even Glavand's recent careful study on the lobster (1948) gave only a rough average value for the normal fibrinogen level in this species. Such information is of interest in evaluating the coagulation function in various invertebrates and in comparing it to that in vertebrates.

Materials and Methods

Animals

Some 150 individuals representing 12 species were collected by hand or trapped in an 8-week period in the summer of 1946. The availability of these various crustaceans is indicated in Table I where the numbers, weight range, sex, habitat and mode of capture of these animals are summarized. All specimens were caught personally except for the spiny lobsters which were taken by commercial fishermen and the locust lobsters which came from the Bermuda Aquarium through the kindness of its director, Mr. Louis Mowbray.

A number of other species were collected but not studied because only one or two specimens were available and/or because the species were too small to allow effective collection of blood. These included the ghost crab (*Ocypode arenarius*), the gulfweed crab (*Planes gracilis*), the flat crab (*Percnon planissimum*), the spider crabs (*Mithrax forceps, Microphrys bicornutus* and *Macrocoeloma trispinosum*), petrolisthes (*Petrolisthes armatus*), the red-hermit crab (*Calcinus sulcatus*),

[1] Contribution No. 190 from the Bermuda Biological Station. We are indebted to the Director, Dr. D. E. S. Brown, for his assistance during this study and to the Porter fund for a grant-in-aid.

TABLE I

General characteristics of the species studied

Species	Weight in gm.	No. ♂	No. ♀	With eggs	Location	Capture
Portunidae						
Callinectes ornatus	50–85	9			Sheltered muddy bay, free swimming or buried	Net or trap
C. danae (Bermuda blue crab)	50–140	3	2			
C. sapidus (common blue crab)	165–380	4				
Achelous smithii	100–115				Sheltered channel or open bay	Trap
Piluminidae						
Eupanopeus herbstii	10–59	17	11	3	Sheltered bay; in or on muddy bottom	Trap or under stones
Grapsidae						
Plagusia depressa (cliff crab)	34–130	10	11	4	Rocky open shore, always in water	Net
Grapsus grapsus (rock crab)	25–105	11	14	6	Rocky open shore, in or out of water	Hand or net
Goniopsis cruentatus (Mangrove crab)	18–43	9	8	2	Mangrove swamp, in or out of water	Trap
Gecarcinidae						
Gecarcinus lateralis (common land crab)	1.8–27	19	18	4	Stony island or beach	Under stones
Paguridae						
*Dardannus venosus** (red-veined hermit crab)	56				Sheltered channel	Trap
Palinuridae						
Panulirus argus (spiny lobster)	150–4500				Reef	Trap
Scyllaridae						
Scyllarides aequinoctialis (locust lobster)	720–870				Reef	Trap

* The only large hermit crab listed for this region. In this specimen, however, the right chela was the larger.

the tri-colored hermit crab (*Clibanarius tricolor*), *Glypturus branneri* and the "split thumbs" (*Gonodactylus oerstedii* and *Pseudosquilla ciliata*). (Nomenclature follows Verrill, 1908, 1922.)

The rock crab (*Grapsus grapsus*) was perhaps the most abundant large crustacean on the island but because of its agility and thin shell it was very difficult to capture uninjured specimens by ordinary means. At night, however, these animals could be approached with a light and picked up directly from the rocks where they lay just above the waterline. By this means 5 were taken in 15 minutes on one occasion and 7 in 25 minutes on another, as compared to 5 animals taken alive in

two hours by two men during the day. These crabs killed one another in a small container or tank and seemed to survive poorly when this was prevented by removing the chelae. In a large tank with a screened enclosure which permitted them to come out of water, they remained healthy and vigorous for several weeks. Another large grapsoid crab with a heavier shell, the "cliff" crab (*Plagusia depressa*), was taken in considerable numbers although it is listed as "rare" by Verrill (1908). Belying its name, it was never seen above water but as many as 10 to 14 were taken from just below the water line along rocky shores on still sunny mornings.

Bleeding

The animals were bled from the limbs after amputation, usually within 4 to 20 hours of capture. Usually the chelae were cut first and then one of the larger walking legs. The courses of two representative bleedings are shown in Figure 1. Although coagulation occurred quickly in the collecting vial, usually within a minute, it was often many minutes before flow stopped at the limb. Bleeding was encouraged by holding the cut stump downward and by maintaining pressure on the carapace. After the flow had stopped at one limb or following autotomy, another was amputated, usually with a renewed flow, but this procedure was seldom effective beyond the second or third limb (Fig. 1). There was characteristically a quite abrupt end-point beyond which no more blood could be obtained from the animal despite the application of pressure, time or further amputation.

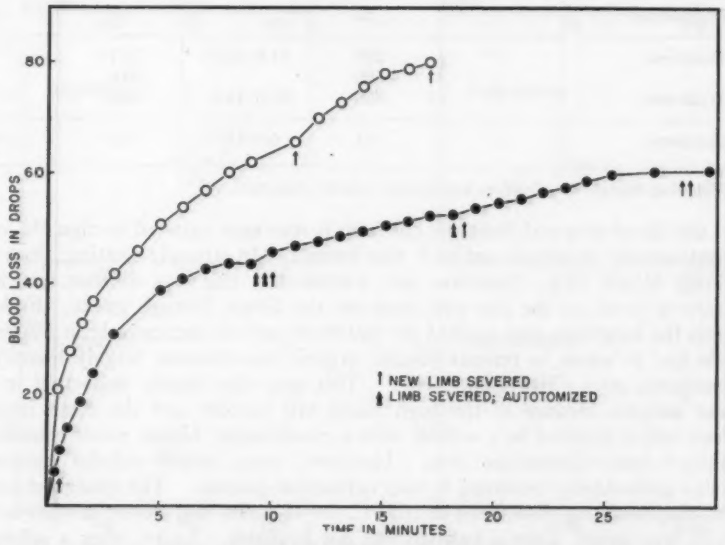


FIGURE 1. Course of bleeding in two representative individuals. Open circles, *Callinectes danae*, 76 g. ♂; bleeding volume 7.0 cc.; average drop, 0.09 cc. Closed circles, *Gecarcinus lateralis*, 27 g. ♂; bleeding volume 3.6 cc.; average drop, 0.06 cc. After an initial rush of blood the bleeding volume was roughly proportional to the square root of the elapsed time ($V = kt^{\frac{1}{2}}$).

Coagulation

The term coagulum will be used to describe the total product of the two processes, the agglutination of cells and the polymerization of fibrinogen into fibrin. In some species this coagulum is largely a cellular agglutinate while in others it is largely a fibrin clot.

TABLE II

The bleeding volume in various Crustacea

Genus	No.	Ave. wt. g.	Bleeding volume in cc./100 g.		
			Range	Mean	σ
Gecarcinus	35	12	5.6–13.9	9.5	2.8
Grapsus	31	72	4.0–10.8	6.6	1.9
Goniopsis	13	25	4.1–10.5	6.9	2.1
Plagusia	28	75	3.3– 8.8	5.5	1.6
(Grapsidae)	72	64		6.2	(27%)
Eupanopeus	25	29	1.3– 7.1	3.8	1.5
Callinectes	13	130	6.9–18.8	10.5	3.2
Achelous	2	108	6.0– 7.0	6.5	—
(Portunidae)	15	127		10.0	(36%)
(*Brachyura*)	147	53		6.9	
Dardannus	1	56		5.0	—
Panulirus	3*	270	(4.0–10.7)	(6.7)	
	1	4,500		20.0	
Scyllarides	2	800	15.3–18.5	16.9	
Cambarus	7	34	6.0–10.9	8.6	2.0

* Bleeding discontinued before maximum volume obtained.

As the blood dropped from the cut limb it was kept agitated so that the coagulum continuously synerized out as it was formed. In strongly clotting, fibrinogen-containing bloods (*e.g., Panulirus* and *Callinectes*) this was difficult and it was necessary to break up the clot and press out the serum through gauze. Following syneresis the coagulum was washed for half-hour periods successively in 1% sodium chloride and in water to remove soluble organic constituents, largely hemocyanin, and inorganic salts (Morrison, 1947). This step was doubly important in some of these animals because of the high blood salt content and the often imperfect syneresis which resulted in a residue with a considerably higher water content than is obtained from mammalian clots. However, some soluble cellular components were also undoubtedly removed by this extraction process. The synerized and extracted coagulum was then dried at 105° C. for 48 hours and stored in vials between layers of lens paper, since a balance was not available. Later, after a subsequent 48-hour drying period the coagulum was weighed to 0.1 mg. In smaller species the blood from several individuals was pooled in order to obtain a sufficient quantity of coagulum.

Results and Discussion

Bleeding volume

The data on bleeding volumes in the several species are summarized in Table II. Individual values within a species varied over a three-fold range and average values ranged from 3.8 in *Eupanopeus* to 16.9 cc./100 grams of body weight in *Scyllarides*. The values for the Macrurans averaged higher (13 per cent) than the Brachyurans (7 per cent) and of the latter the swimming Portunids averaged somewhat higher (10 per cent) than the others (6.5 per cent). Part of this latter difference undoubtedly derives from the fact that the climbing or crawling crabs bear a considerably heavier weight of shell than do the lighter swimming or walking species. Thus, values for the per cent of body water ranged from 53 and 54 in *Plagusia* and *Eupanopeus* to 76 and 78 in *Callinectes* and *Gecarcinus*. Bleeding volumes expressed as per cent of the body water were, with the exception of *Eupanopeus* (7.0), much more constant, ranging from 10.4 in *Plagusia* to 13.8 in *Callinectes*.

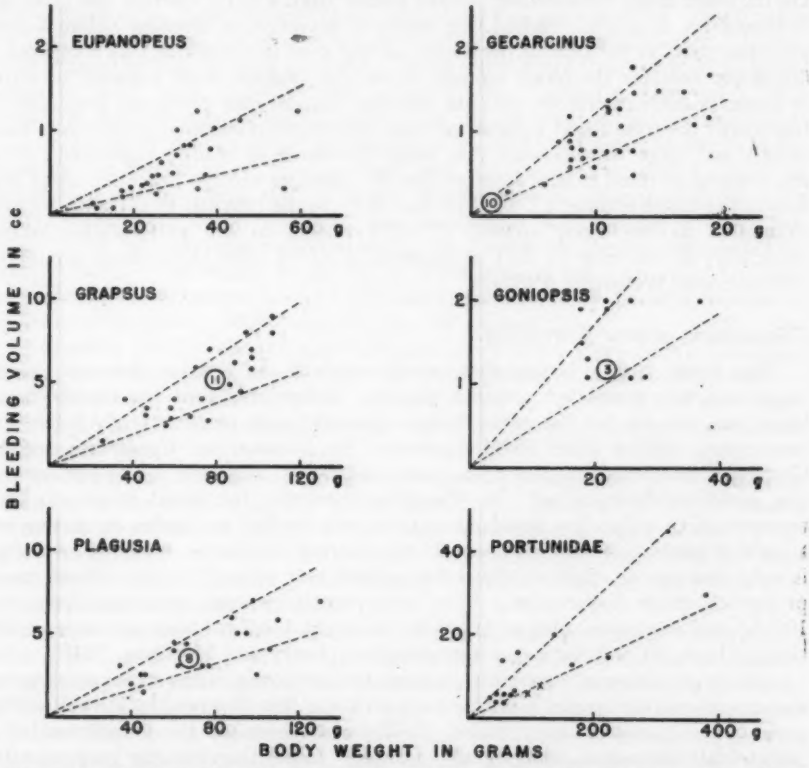


Figure 2. Bleeding volume in 6 genera of Crustacea as a function of body weight. Symbols show individual values except the circles which represent pools of the indicated size. Broken lines show standard deviations.

Bleeding volumes for 6 of the species are plotted as a function of body weight in Figure 2. Some tendency toward lower per cent yields in the smaller individuals is seen in *Grapsus* and particularly in *Eupanopeus,* and this may in part account for low bleeding volume in the latter species. A similar group correlation with weight may be seen since the heavier Macrurans have the largest bleeding fraction, the Portunids are next and the smaller Brachyurans are last. This correlation breaks down in comparing the three smallest genera, however, since of these the lightest, *Gecarcinus* (12 g.), has the largest bleeding fraction followed by *Goniopsis* (25 g.) and *Eupanopeus* (29 g.).

These crustacean bleeding fractions of 4 to 17 compare to a total blood volume of 25 cc./100 g. as measured in crayfish (*Cambarus virilis*) by Prosser and Weinstein (1950), using dye dilution methods with T-1824 or thiocyanate. Measurements of the bleeding volume on this same species gave values ranging from 6 to 11 and averaged 8.6 cc./100 g. (Table II). Since this value is only one-third of that obtained by dye dilution, it is evident that much blood is left behind in bleeding. On the other hand, the bleeding volume results from a fairly discrete end point and is reasonably constant. Indeed, the standard deviation of bleeding volumes averaged less than 30 per cent of the mean (20 per cent in *Cambarus*) as compared to 36–38 per cent for the blood volume in the dye dilution study referred to above. It seems possible, therefore, that the bleeding volume may represent some definite fraction of the total blood volume, perhaps that fraction occupying the heart, blood vessels and large sinuses since this would be the most readily expressed. With this concept in mind it is of interest that the bleeding volume bears the same relation to the blood volume (T-1824 or NaCNS) in the crayfish (1:3) with its open circulation as the blood volume (T-1824) bears to the extracellular volume (NaCNS) in the frog (1:3.3) or mammal (1:3–4) with their closed circulation (Prosser and Weinstein, 1950).

Coagulation; general description

The initial step in coagulation, which removed the cellular elements, usually began and was completed within a minute. Before this time the freshly drawn blood was opaque but thereafter it was clear although often strongly colored by hemocyanin and/or other blood pigments. In *Eupanopeus, Gecarcinus* and the Grapsidae this initial coagulum was fairly substantial and little if any further clotting could be distinguished. In *Panulirus,* however, the initial stage was often represented by only a few shreds of material and the first intimation of clotting was a sudden gelation of the solution. If the clotting solution is undisturbed the gel is very transparent, rigid, elastic and synerized only with difficulty. These are all properties which characterize a "fine" clot containing small structural fibers (*ca.* 100 Å) and interstices such as is formed from mammalian fibrinogen under conditions of high pH and fibrinogen concentration (Ferry and Morrison, 1947).

Under physiological conditions mammalian fibrinogen forms a coarser structure than crustacean fibrinogen, a distinction which was first observed by Howell (1916) under the ultramicroscope. Stubel (1920) also described the transformation of invertebrate fibrinogen as into small "granules" rather than into the long "needles" characteristic of mammalian material. In the amphibians and fishes, however, fine structured clots are seen in both gross and microscopic observation (Stubel,

1914). Among the vertebrates this difference in clot structure may be correlated with differences in the solubilities of the respective fibrinogens (Morrison, Scudder and Blatt, 1951). The more soluble fibrinogens found in the lower vertebrates may represent smaller, less asymmetric and/or more highly charged molecules, any or all of which conditions could account for the "finer" clots formed from them. That a similar situation may exist in crustacean fibrinogen is further suggested by the observation of Glavand (1948) that a relatively high gel fraction (*ca.* 1.5 g./1.) was necessary for clotting in these animals. A gel fraction of as little as 0.05 g./1. is sufficient to form a continuous clot in systems with mammalian fibrinogen (Ferry and Morrison, 1947).

Coagulum weights

Average weights for the coagula are summarized in Table III. Values by species ranged from 3.6 g./1. in *Plagusia* to 16.6 g./1. in *Panulirus*. Individual values are very variable, with standard deviations averaging about 50 per cent and with as much as an eight-fold difference being observed in *Callinectes*. Such variation in the coagulating ability of crustacean blood has been noted before. Cuénot (1891) reported that strength of the clot varied much more than the level of hemocyanin in blood from starving crabs and lobsters. Similarly, Glavand (1948) found that the fibrinogen in lobster blood often disappeared entirely during captivity. In this study the two locust lobsters had been held in captivity in the Bermuda Aquarium for a number of months which may account for the smaller coagulum, negligible in one specimen, and the longer clotting time as compared to the spiny lobsters which were freshly trapped. Similar changes in nutritional or other conditions in the natural trapped specimens may be responsible for the high variability observed here. The natural variability of fibrinogen in the lady crab, *Ovalipes*, was apparently so great that Loeb (1903) first reported it to be absent in this species and then subsequently (1904) reported it to be present.

Gruzewska (1932) related lower fibrinogen levels in the green crabs (*Carcinides maenas*) to moulting. Values for the only two soft-shelled (blue) crabs taken in this study were not included in the average values in Table III. These values (2.4 and 2.5 g./1.) are among the lowest found in this group being less than half of the mean value.

The Macrurans formed much heavier coagula (average = 17 g./1.) than the Brachyurans (average = 5 g./1.). This agrees with the qualitative observations of much more effective clotting in European Macrurans as reported by Cuénot (1891) (*Palinurus*), Heim (1892) (*Palinurus, Homarus*), Tait (1911) (*Palinurus, Homarus* and *Astacus*), and Glavand (1948) (*Homarus* and *Nephrops*), and in *Homarus americanus* by Loeb (1904).

Large differences in clotting ability are found among the various Brachyurans. Heim (1892) and Tait (1911) both mention *Portunus puber* as the one species among many crabs which approaches the Macrurans in this regard and Loeb (1904) and George and Nichols (1948) refer to the firm clots in the related *Callinectes*. In Table III it may be seen that the Portunidae do average somewhat higher (5.8 g./1.) than the Grapsidae (4.0 g./1.) but because of the high variability the difference is barely significant ($t = 2.7$). Of more interest, and correlating with the very firm clots often observed in this group, may be the fact that the highest indi-

TABLE III

Coagulum weights in various Crustacea

Genus	No.*	Coagulum in g./l.			Clotting time** sec.
		Range	Mean		
*Gecarcinus****	10	2.1– 5.6	3.6	1.2	44
Grapsus	17	2.6– 6.2	4.1	1.2	
Goniopsis	5	2.5– 6.3	4.0	2.1	
Plagusia	3	3.0– 4.3	3.6	0.9	35–120
(Grapsidae)	25		4.0	(33%)	
Eupanopeus	7	3.0– 7.2	4.7	1.8	5–90
C. ornatus	8	1.5–13.0	5.0	4.7	60
C. sapidus	3	5.0– 7.4	6.2	1.5	60
C. danae	3	2.5– 9.8	8.6	1.6	60
Achelous	2	2.1– 5.5	3.8	2.8	52–75
(Portunidae)	16		5.8	(43%)	
(*Brachyura*)	63		4.8		
Dardannus	1		15.6		
Panulirus	7	8.6–22.3	16.6	5.0	
Scyllarides	2	(1.8– 8.9)	(5.3)		300
*Cambarus*****	5	1.1– 3.3	2.1	1.0	40–65

* Each value represents a pool of 1 to 6 individuals.

** Blood delivered from limb into a glass vial; time measured to the first appearance of a clot; values represent 1 to 3 observations.

*** Excluding value of 1.5 g./l. for a pool from 10 very young (average weight = 1.8 g.) individuals.

**** Commercially supplied individuals from Wisconsin. Time after capture not known.

vidual values (to 13.0) were found in the Portunidae. Only one Anomuran species was studied. The single *Dardannus*, a large hermit crab, had a higher value (15.6 g./1.) than any of the Brachyurans. This may correlate with the lesser defense against injury provided by its soft body.

Agglutination vs. clotting

In this study measurements of fibrinogen alone were not made but only of the extracted coagulum which also contained cellular components. One might estimate the fibrinogen level in those animals possessing it by subtracting from the weight of the coagulum, an amount equal to that in those species which are considered not to have fibrinogen. But a simple correction of this sort is of questionable validity since it assumes that the various species have comparable amounts of cellular material. This is certainly not the case since Yeager and Tauber (1935) found cell counts ranging from 5×10^3/mm.^{3} in *Talorchestia* to 54×10^3/mm.^{3} in *Callinectes* (average = 18×10^3/mm.^{3} in 13 species of crustaceans) with an individual standard

deviation of 30 to 60 per cent. Similarly, George and Nichols (1948) found a hematocrit value of 1.0 per cent in *Callinectes* but only 0.25 per cent in *Libinia*. The species with distinct fibrinogen clotting also appear to have more cells. Although these additional cells do not necessarily represent agglutinating components they will nonetheless become enmeshed in the clot structure and contribute to the weight of the coagulum. Still, even in *Callinectes*, the form most abundantly supplied with cells, the dry cell weight presumably amounts to only 20–25 per cent of the cell volume or 2–2.5 g./l. and the extracted dry weight should be even less. In *Libinia* and other species with fewer cells, a value of only 1/2 to 1/4 the above or 0.5–1.2 g./l. is expected. Since average values of about 4 g./l. (3.6–4.6 g./l.) or 3 to 8 times the above estimate were found even in the five lowest species in Table III, it would appear that considerable material had been adduced to the cellular components from the plasma even in those species which show only an "initial" coagulation phase and which formerly had been considered to have no fibrinogen in their plasma. However, although this unidentified material precipitates from plasma on the shedding of blood it should perhaps indeed not be considered as fibrinogen since it does not form the gel structure which we are accustomed to associate with fibrin.

Using a correction of 1–2 g./l., the dry cell weight estimated above, we arrive at a mean value of 3 g./l. for the plasma component in *Eupanopeus, Gecarcinus* and the Grapsidae. In the spiny lobster, *Panulirus*, the fibrinogen value is five times this amount or 15 g./l. and individual values range up to 20 g./l. Glavand (1948) reported values for the fibrinogen level in the common lobster, *Homarus vulgarus*, ranging from 2.9 to 10.9 g./l. and stated (p. 96) that "fibrinogen of about 0.4 per cent (4 g./l.) in the citrated plasma is as a rule found." These values are considerably lower than those in the spiny lobster but the Palinuridae, *i.e., Palinurus*, have been previously cited as having the most strongly clotting blood of any of the crustacea, including the other Macrurans (Tait, 1911).

Mammalian vs. crustacean fibrinogen levels

It is of considerable interest to compare the fibrinogen level in mammals to that in these crustaceans. The clot formed by the blood of *Panulirus* is certainly equal and probably superior to an ordinary mammalian clot in strength and rigidity. However, this appears to be achieved only by the use of a considerably larger amount of material (15 g./l.) since the normal fibrinogen level in mammals is at only 2–4 g./l., although in some species, *e.g.*, swine and cattle, values up to 8–10 g./l. have been reported. In the less effective of the crustaceans the coagulum weight was roughly equal to the fibrinogen level in many mammals, but in functional terms of gel formation the two groups cannot even be compared.

In terms of variability a striking contrast is seen between the mammals and the crustaceans. Gram (1921) in measurements on 50 men and women found an average value of 2.8 g./l. with a *range* of ± 30 per cent. Similarly, Ham and Curtis (1938) found an average value of 2.5 g./l. with a *range* of ± 28 per cent in 38 normal men and women. These values of ± 30 per cent for the *range* of variation in sizable groups of men compare with *standard deviations* of about 40 per cent in the Crustacea. The latter value is low since a number of the individual values upon which it was based represented pools of blood from up to six individuals. The difference is even more striking in single individuals which in

the crustaceans can lose all coagulation function (Glavand, 1948) but which in man vary by only ± 4–8 per cent from the mean over periods of more than a year (Gram, 1921; Ham and Curtis, 1938). It should be noted, however, that fibrinogen levels of up to 7 g./l. have been recorded in man in response to a variety of infectious diseases (Ham and Curtis, 1938).

Coagulation and hemostasis

Some inverse correlation between autotomy and clotting ability may be seen in the fact that the heaviest clots came from a genus (*Panulirus*) in which it is difficult to induce the autotomy of even a single limb while the lightest clots came from genera (*Gecarcinus, Eupanopeus* and the Grapsidae) which are very prone to autotomy. Tait (1911) considered this proposition, *i.e.*, that these two hemostatic mechanisms might substitute for one another, and although most of his data

TABLE IV

*Hemostatic influences in various arthropods**

Genus	Size	Shell	Autotomy	Cells	Fibrin	Σ+	Reference**
Limulus	+	++	0	++++	0	7	d
Libinia	++	++++	+	+	0	8	d,g
Maia	(++)	+	+++	+	0	7	b,g
Gecarcinus	+++	+	++++	+	0	9	a,g
Goniopsis	+++	++	+++	+	(0)	9	a
Plagusia	++	++++	++	+	(0)	9	a
Grapsus	++	+++	++	+	(+)	9	a
Eupanopeus	+++	++	++	++	0	9	a,g
Cancer	++	+++	+	+	+	8	e,f,g
Carcinides	++	++	++	+	+	8	e,f,g
Callinectes	++	++	++	+	++	9	a,d,g
Portunus	++	++	++	+	+++	10	c,e,g
Dardanus	+++	+	(+)	?	+++	8	a
Astacus	+++	++	+	0	+	7	e,c,f,g
Cambarus	+++	++	+		+	7	a,g
Homarus	+	+++	+	+	++	8	d,f,g
Scyllarides	+	++	++		++	7	a
Panulirus	+	+++	+	(0)	+++	7	a
Palinurus	+	(++)	++	0	+++	8	b,c,e,g
Isopods	+++	+	0	?	+++	(7)	e

* Average adult size as *small* (< 60 g.), +++; *medium* (60–300 g.), ++; or *large* (> 300 g.), +. Shell weight as *light* or *absent*, +; *moderate*, ++; *heavy*, +++; or *very heavy*, ++++. Tendency toward autotomy as *very strong*, ++++; *strong*, +++; *moderate*, ++; *weak*, +; or *absent*, 0. "Cellular agglutinate" (initial coagulum) or fibrin clot as *heavy*, +++; *moderate*, ++; *light*, +; or *negligible*, 0.

** References: a, this study; b, Cuénot (1891); c, Heim (1892); d, Loeb (1903); e, Tait (1911); f, Glavand (1948); g, Wood and Wood (1932).

were affirmative he felt that two notable exceptions invalidated the principles. Possibly he attempted too great a simplification since fibrinogen and autotomy are not the only factors which affect the potential loss of blood in crustaceans. The agglutinating cellular constituents certainly facilitate hemostasis and the strength of the shell must be important in preventing injuries which might cause blood loss. The over-all size of the individual may also be important since a given thickness of shell will afford less protection to a large animal than a small one. Further, bleeding from a large limb would be more diffcult to staunch than from a small limb. Indeed, in the smaller Crustacea it was often not possible to obtain even a drop of blood from the severed appendage.

The interrelations of these several factors in a number of arthropods are summarized in Table IV where arbitrary grades of intensity have been assigned to the several quantities. The sums of these various values are tabulated in column 6 and show reasonable constancy for the various species. Thus, while the spider crab, *Libinia,* has neither fibrinogen nor effective power of autotomy it is very strongly protected by its shell. *Gecarcinus,* on the other hand, which has protection from neither shell nor fibrinogen has a highly developed power of autotomy. Again, the spiny lobster with little capacity for autotomy and not too heavy a shell in relation to its large size possesses a high concentration of fibrinogen while *Limulus,* with similar disadvantages, compensates by a copious cellular agglutination. Other crabs, *Callinectes, Carcinides* and *Grapsus,* in contrast, appear to utilize all of the mechanisms but each in moderate extent. In brief, then, hemostasis appears to be an important function in these animals, but one which may be accomplished either singly or in concert by a variety of very different means.

Summary

1. Maximum bleeding volumes and coagulum weights were determined on more than 150 individuals of 12 species of Bermudan crustaceans.

2. Average values for the bleeding volume ranged from 3.8 cc./100 g. in *Eupanopeus* to 16.9 cc./100 g. in *Scyllarides* and averaged 7.2 cc./100 g., an amount equal to roughly one-third of the true blood volume in crustaceans but about equal to the blood volume in vertebrates.

3. Average values for the weight of the extracted and dried coagulum representing the sum of fibrin and cellular constituents ranged from 3.6 g./l. in *Plagusia* to 18.1 g./l. in *Panulirus* with large individual variation in most species.

4. Even in animals previously considered to possess only an "initial," cellular phase of coagulation, the coagulum weights were 3–8 times greater than the estimate of the maximum cellular contribution and it was concluded that plasma components must be of importance in these species.

5. In species possessing considerable amounts of fibrinogen a "fine-structured" clot is formed resembling that in lower vertebrates rather than that in mammals. The mechanical properties of this clot compare favorably to the mammalian clot but 3–6 times as much fibrinogen is employed.

6. A comparison of species with regard to the various factors modifying hemostasis, *i.e.,* fibrinogen, agglutinating cells, autotomy, size, and shell strength shows a compensation whereby animals lacking one or more mechanisms were particularly strong in others.

LITERATURE CITED

CUÉNOT, L., 1891. Études sur le sang et les glandes lymphatiques dans la série animale. *Arch. de Zool. Exper. et Gen.*, 2nd series, **9**: 13–90.

FERRY, J. D., AND P. R. MORRISON, 1947. The conversion of human fibrinogen to fibrin under various conditions. *J. Amer. Chem. Soc.*, **69**: 388–400.

GEORGE, W. C., AND J. NICHOLS, 1948. A study of the blood of some Crustacea. *J. Morph.*, **83**: 425–443.

GLAVAND, J., 1948. Studies on the coagulation of crustacean blood. Thesis, Copenhagen.

GRAM, H. C., 1921. A new method for the determination of the fibrin percentage in blood and plasma. *J. Biol. Chem.*, **49**: 278–295.

GRUZEWSKA, Z., 1932. Sur la coagulation du sang chez les crustacés. *C. R. Soc. de Biol.*, **110**: 920–922.

HAM, T. H., AND F. C. CURTIS, 1938. Plasma fibrinogen response in man. *Medicine*, **17**: 413–445.

HEIM, F., 1892. Études sur le sang des crustacés decapodes. Thesis, Paris (cited by Glavand).

HOWELL, W. H., 1916. Structure of fibrin-gel and theories of gel-formation. *Amer. J. Physiol.*, **40**: 526–546.

LOEB, L., 1903. Über die Bedeutung der Blutkörperchen für die Blutgerinnung and die Entzündung einiger Arthropoden und über mechanische Einwirkungen auf das Protoplasma dieser Zellen. *Virchow's Arch. f. Pathol. Anat. u. Physiol.*, **173**: 35–112.

LOEB, L., 1904. Über die Koagulation des Blutes einiger Arthropoden. *Beitr. Z. Chem. Physiol. u. Pathol.*, **5**: 191–207.

MORRISON, P. R., 1947. Some factors influencing the quantitative determination of fibrinogen. *J. Amer. Chem. Soc.*, **69**: 2723–2731.

MORRISON, P. R., C. SCUDDER AND W. BLATT, 1951. The solubilities of some vertebrate fibrinogens in plasma-ethanol mixtures. *Biol. Bull.*, **101**: 171–177.

PROSSER, C. L., AND S. J. F. WEINSTEIN, 1950. Comparison of blood volume in animals with open and closed circulatory systems. *Physiol. Zool.*, **23**: 113–124.

STUBEL, H., 1914. Ultramikroskopische Studien über Blutgerinnung und Thrombocyten. *Pflüger's Arch. f. d. ges. Physio.*, **156**: 361–400.

STUBEL, H., 1920. Die Fibringerinnung als Krystallisationsvorgang. *Pflüger's Arch. f. d. ges. Physiol.*, **181**: 285–309.

TAIT, J., 1911. Types of crustacean blood coagulation. *J. Marine Biol. Assoc.*, **9**: 191–198.

VERRILL, A. E., 1908. Decapod Crustacea of Bermuda. I. Brachyura and Anomura. *Trans. Connecticut Acad. Arts and Sci.*, **13**: 299–494.

VERRILL, A. E., 1922. Decapod Crustacea of Bermuda. II. Macrura. *Trans. Connecticut Acad. Arts and Sci.*, **26**: 1–179.

WOOD, F. D., AND H. E. WOOD, 1932. Autotomy in decapod Crustacea. *J. Exp. Zool.*, **62**: 1–55.

YEAGER, J. F., AND O. E. TAUBER, 1935. On the hemolymph cell counts of some marine invertebrates. *Biol. Bull.*, **69**: 66–70.

RESPIRATORY METABOLISM OF AMPHIBIAN GASTRULA EXPLANTS

NORMA ORNSTEIN AND JOHN R. GREGG

Department of Zoology, Columbia University, New York 27, New York

Chemical embryologists suppose that familiar differences between the *morphological* behavior of the dorsal side of an amphibian gastrula and that of its ventral side are associated with causally significant differences in their *metabolic* behavior. But numerous attempts to confirm this plausible hypothesis have yielded results that are somewhat disappointing. Several items contribute to this undesirable state of affairs: crucial technical difficulties in handling gastrula explants are only now coming to be explicitly recognized; there is a seeming reluctance among chemical embryologists to repeat the work of others, so that probably unwarranted conclusions are currently accepted; and, worst of all, even the most carefully obtained results have not been suggestive of really fruitful explanatory hypotheses.

Nevertheless, there seems to be open no course other than to continue piecemeal the biochemical exploration of gastrulae in the hope that useful information will be obtained. In accordance with this view, we have (a) reinvestigated the confused question whether or not there is a dorso-ventral differential in respiratory rate in amphibian gastrulae, (b) studied the respiratory response of gastrulae tissues to the action of metabolic poisons that suppress gastrulation, in the expectation that dorsal and ventral explants might respond differently, and (c) tried to confirm the interesting report of Boell, Needham and Rogers (1939) that anaerobic carbon dioxide production of dorsal explants is about triple that of ventral ones.

Methods

Explants

Rana pipiens embryos at Stage 10 (dorsal lip; Shumway, 1940) were dissected in full-strength Holtfreter's solution with the aid of glass needles. Each dissected gastrula yielded four explants (Fig. 1); dorsal-left (D,L), dorsal-right (D,R), ventral-left (V,L) and ventral-right (V,R). A serious attempt was made to obtain explants symmetrically located with respect to the dorso-ventral and animal-vegetal axes. Loose yolk cells were removed before and after the explants were healed.

Gas exchange measurements

Measurements of respiratory rate (QO_2: μl oxygen/mg. dry weight of tissue hour at 22° C.) were made with the help of silicon-coated cylindrical Cartesian divers (Holter, 1943) whose average total volume was about 17 μl. Each diver contained the following items:

Two explants of the same class: (D,L) or (D,R) or (V,L) or (V,R)
Medium for explants (full-strength Holtfreter's, no bicarbonate): 0.75 μl
NaOH seal: 1.25 μl
Oil seal: 0.9 μl
Mouth seal: 2.5 μl

With these divers, and employing as a test system autoxidizing cysteine, oxidation rates varying between 0.01 and 0.05 μl/hour could be estimated with a standard deviation of 0.002 (25 experiments).

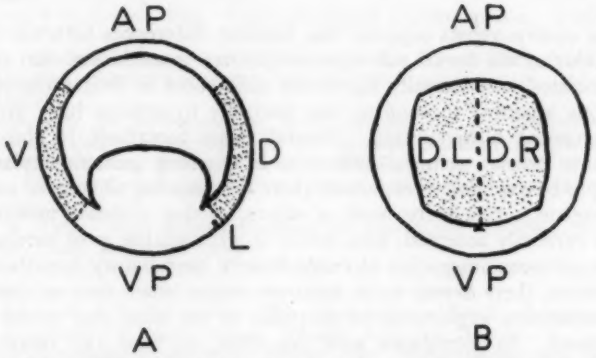


FIGURE 1. A. Diagram of medial sagittal section of a Stage 10 gastrula. Stippled areas show positions from which dorsal (D) and ventral (V) explants were taken. B. Dorsal view of Stage 10 gastrula, showing how dorsal explant is cut into left (D,L) and right (D,R) parts. The ventral explant is similarly treated. Other abbreviations: AP = animal pole; VP = vegetal pole; L = dorsal lip.

Using the same divers, anaerobic carbon dioxide production ($Q_{an}CO_2$: μl carbon dioxide produced/mg. dry weight of tissue/hour at 22° C.) was measured by a method essentially that of Boell, Needham and Rogers (1939). Each diver contained:

Two explants of the same class
Medium for explants (0.03 *M* bicarbonate in full-strength Holtfreter's solution): 0.75 μl
Oil seal: 0.9 μl
Mouth seal: 3.4 μl

Anaerobiosis was insured by the following procedure. After introducing explants into a diver (see below) it was mounted in such a way that it was held upright in a spring clamp with its mouth about two centimeters below the surface of some 95% flotation medium (without taurocholate) contained in a glass cylinder mounted on a vertically-moving rack and pinion (Fig. 2). A stream of gas (95 parts N_2: 5 parts CO_2, previously de-oxygenated over hot copper and washed with water) was then led into the diver with a capillary pipette (as shown) for at least one minute. The diver was then moved along to a second pipette which was used to

place the oil seal. The space above the oil seal was next re-gassed for about fifteen seconds, and the diver moved along to a third pipette, which was used to suck out gas from above the oil seal until by test the diver barely stayed afloat in the medium in the cylinder. This latter procedure automatically introduced a mouth seal of the proper volume.[1]

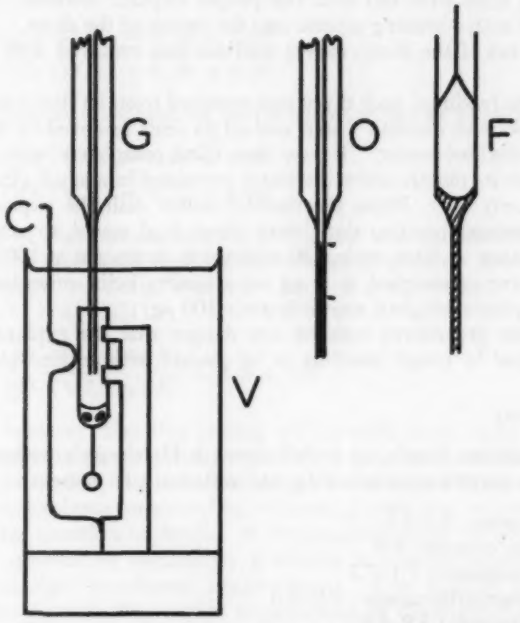


FIGURE 2. Diagram of apparatus used to fill divers anaerobically (not to scale). Diver with two explants shown held by spring clamp (C) in glass vessel (V) filled with flotation medium. Gas pipette (G) in position to gas diver. (O), pipette filled with oil, ready to place oil seal. Two marks represent calibration marks. (F), braking pipette partly filled with flotation medium, ready to insert into diver and remove gas from above oil seal, thus placing mouth seal.

We believe that nearly complete anaerobiosis is thus achieved. For example, a cysteine solution was prepared, samples of which autoxidized under iron catalysis in air-filled divers at the rate of 0.105 μl/hour, whereas control samples in anaerobic divers exhibited a gas uptake of only 0.0009 ± 0.0018 μl/hour (3 experiments). Obviously, oxygen uptake in the anaerobic divers is not significantly different from zero.

When the divers were filled, they were at once transferred to the diver flotation vessels in a constant temperature bath (22° C.) and equilibrated for at least one

[1] This apparatus was kindly lent to us by Dr. L. C. Sze. He used it by filling the cylinder with distilled water kept anaerobic by bubbling the $N_2 : CO_2$ mixture through it. The practice of using flotation medium, in which oxygen is negligibly soluble, is our own innovation.

half hour before readings were begun. Readings were generally made for two hours at intervals of from 10 to 15 minutes.

Loading explants into divers, removing and weighing

Explants were easily and safely loaded into the divers in the following manner. Each diver was filled over-full with the proper explant medium. Explants were then transferred with a braking pipette into the mouth of the diver. When they had sunk to the bottom of the diver, excess medium was removed with a fine capillary pipette.

Following the readings, each diver was removed from its flotation vessel, washed off on the outside with distilled water, and all its seals removed by flushing its neck copiously with distilled water. It was then filled completely with distilled water, and inverted with its mouth under the water contained in a small glass dish until the explants fell slowly out. From the distilled water dish the explants were transferred with a braking pipette, along with about 5 μl water, to small pre-weighed bits of cigaret paper. After drying 30 minutes to overnight at 100° C., the papers and explants were re-weighed to 1 μg on a quartz helix microbalance. On the average, two explants weighed approximately 100 μg.

None of these procedures entailed any danger that the explants would be injured or destroyed by rough handling or by contact with an air-water interface.

Inhibitor solutions

Inhibitor solutions (made up in full-strength Holtfreter's without bicarbonate) employed in the experiments following had different pH values:

Sodium azide: 6.3–6.4
Potassium cyanide: 9.8
Sodium malonate: 7.1–7.3
p-chloromercuribenzoate: 7.9–9.8
Sodium fluoride: 5.8–5.9
2,4 dinitrophenol: 5.0–5.1

Control explants were run in Holtfreter's adjusted with NaOH or HCl to the pH of the corresponding inhibitor solutions. A survey of the results will show that respiratory activity of explants was the same in all these control solutions as in plain Holtfreter's. Furthermore, whole gastrulae develop normally in all of them.

Throughout the inhibitor studies reported in this paper, for each experimental (control) explant used, there was a corresponding control (experimental) explant *from the same gastrula.*

To maintain cyanide concentrations around the explants in the divers (pH 9.8) it was necessary to add cyanide to the NaOH seal as follows:

KCN around explants	*KCN in NaOH seal*
0.0001 *M*	0.09 *M*
0.0005 *M*	0.44 *M*

(*cf.* Umbreit, Burris and Stauffer, 1945; p. 45 ff.).

Results

Respiration in full-strength Holtfreter's solution

In Holtfreter's solution, which for the purpose of these investigations we shall consider to constitute a relatively normal environment for explants, lateral halves of dorsal explants respire at about the same rate ($P > 0.05$):[2]

(1) QO_2 (D,L) $= 0.30 \pm 0.08$ (20)[3]

(2) QO_2 (D,R) $= 0.29 \pm 0.05$ (14)

A similar result has been obtained for lateral halves of ventral explants ($P > 0.05$):

(3) QO_2 (V,L) $= 0.25 \pm 0.05$ (19)

(4) QO_2 (V,R) $= 0.25 \pm 0.08$ (14)

A further result of these measurements is that we have confirmed the reports of those investigators who have found a dorso-ventral gradient in respiration with respect to dry weight, for dorsal explants respire at a significantly higher rate than ventral explants[4] ($P < 0.001$):

$$(5)\quad \frac{QO_2 \text{ (D,L and R)}}{QO_2 \text{ (V,L and R)}} = 1.2 \pm 0.2 \qquad (31)$$

It is not likely, however, that this finding will provide much basis for speculation about the relations of respiratory rate to gastrulation, for it seems that when respiratory rates are expressed in terms of some non-yolk reference component, the respiratory rate of dorsal explants comes out to be identical with that of ventral ones. We do not discuss this question in detail. A forthcoming paper by Sze carefully analyzes the whole problem of respiratory gradients in amphibian gastrulae, and his results shed much light on current disagreements. Our findings are quite in line with his. It thus appears that earlier expectations of discovering embryologically significant differences in the respiratory rates of various gastrula-parts under normal conditions are coming to be less and less warranted by new results. Nevertheless there remains the possibility that differential respiratory responses to less physiological environments may yet be obtained. To this question we not turn.

Respiration in sodium azide (NaN_3)

Sodium azide is thought to act upon biological systems by inactivating the cytochrome oxidase system as cyanide does, or by interfering with the action of some enzyme systems involved in anaerobic phosphate transfer. Azide is a powerful inhibitor of gastrulation in amphibian embryos: both Barnes (1944) and Spiegelman and Moog (1945) have reported the failure of *Rana pipiens* embryos to gastru-

[2] For calculating P-values, Student's t-test has been used throughout.

[3] Parenthesized numerals to the extreme right of equations (1)—(65) indicate the number of experiments performed.

[4] In considering quotients such as (5) it may be of help to know that throughout this paper they are calculated only from the results of experiments in which for each dorsal (ventral) explant used there was a corresponding ventral (dorsal) explant *from the same gastrula.*

late in weak concentrations of azide; and our results confirm theirs, for gastrulae placed at Stage 10 in 0.0005–0.001*M* sodium azide do not develop past Stage 10+. Barnes showed also that the respiratory rate of whole gastrulae is sharply reduced (80–90%) in 0.01 *M* azide. But the respiration of gastrula tissues is sensitive even to weaker concentrations of azide.

In 0.0005 *M* azide, for example, dorsal explants respire at a significantly lower rate than their controls ($P < 0.01$):

(6) QO_2 (D,R) 0.0005 *M* $NaN_3 = 0.08 \pm 0$ (3)

(7) QO_2 (D,L) control $= 0.23 \pm 0.08$ (3)

There is a similar reduction of the respiration of ventral explants ($P < 0.001$):

(8) QO_2 (V,R) 0.0005 *M* $NaN_3 = 0.06 \pm 0.02$ (3)

(9) QO_2 (V,L) control $= 0.20 \pm 0.05$ (3)

But there is no indication that the relative rates of respiration of dorsal and ventral tissues are changed by this concentration of azide ($P > 0.05$):

(10) $\dfrac{QO_2 \text{ (D,R) } NaN_3}{QO_2 \text{ (V,R) } NaN_3} = 1.4 \pm 0.2$ (3)

(11) $\dfrac{QO_2 \text{ (D,L) control}}{QO_2 \text{ (V,L) control}} = 1.1 \pm 0.1$ (3)

Analogous results have been obtained with 0.001 *M* azide. The respiration of dorsal explants is greatly reduced ($P < 0.001$):

(12) QO_2 (D,R) 0.001 *M* $NaN_3 = 0.09 \pm 0.02$ (3)

(13) QO_2 (D,L) control $= 0.32 \pm 0.04$ (3)

and so is that of ventral explants ($P < 0.001$):

(14) QO_2 (V,R) 0.001 *M* $NaN_3 = 0.10 \pm 0.02$ (3)

(15) QO_2 (V,L) control $= 0.29 \pm 0.04$ (3)

But, although statistical analysis indicates a probably significant alteration in the relative rates of respiration of dorsal and ventral explants ($P < 0.05$):

(16) $\dfrac{QO_2 \text{ (D,R) } NaN_3}{QO_2 \text{ (V,R) } NaN_3} = 0.87 \pm 0.22$ (3)

(17) $\dfrac{QO_2 \text{ (D,L) control}}{QO_2 \text{ (V,L) control}} = 1.1 \pm 0.2$ (3)

in view of the results obtained with 0.0005 *M* azide ((6)–(11)) we do not consider this established.

To sum up these results with azide, it appears that if gastrulation of *Rana pipiens* embryos is blocked by a specific action of azide, then either this action is not localized in dorsal tissues or else it is one that respiratory measurements do not reveal.

Respiration in potassium cyanide (KCN)

Cyanide, which inhibits respiration in biological systems by blocking cytochrome oxidase electron transfer, is also a strong suppressor of morphogenetic movements in amphibian embryos. Barnes (1944), for example, found that early gastrulae placed in 0.001–0.002 *M* KCN developed only into mid-gastrulae; 0.0001 *M* KCN only slowed gastrulation. Spiegelman and Moog (1945) partially confirmed Barnes's results: in 0.001 *M* KCN, embryos immersed at Stage 10 developed into late gastrulae (Stage 12); while in 0.005 *M* KCN, embryos immersed at Stage 10 remained at that stage. Our experiments indicate variable results with 0.0005 *M* KCN ranging all the way from complete inhibition of invagination to slight slowing only; while with 0.005 *M* KCN, early gastrulae never get past Stage 11.

Barnes also made some measurements of the effect of KCN on the oxygen uptake of whole embryos. She found a maximal effect at concentrations of 0.001 *M*, at which respiration of gastrulae was inhibited about 98%, with graded lesser effects at lower concentrations.

Our results are partly confirmatory of hers. In 0.0001 *M* KCN the respiration of dorsal explants is reduced by a probably significant amount ($P < 0.05$):

(18) QO_2 (D,R) 0.0001 *M* KCN $= 0.20 \pm 0.02$ (2)

(19) QO_2 (D,L) control $= 0.31 \pm 0.05$ (2)

There is a similar effect on ventral explants ($P = 0.01$):

(20) QO_2 (V,R) 0.0001 *M* KCN $= 0.15 \pm 0$ (2)

(21) QO_2 (V,L) control $= 0.27 \pm 0.04$ (2)

Statistically speaking, the effect is probably differential ($P < 0.05$):

(22) $$\frac{QO_2 \text{ (D,R) KCN}}{QO_2 \text{ (V,R) KCN}} = 1.4 \pm 0.1$$ (2)

(23) $$\frac{QO_2 \text{ (D,L) control}}{QO_2 \text{ (V,L) control}} = 1.1 \pm 0.1$$ (2)

But in view of the variable results on whole embryos with this extremely weak solution, and in view of the fact that stronger solutions give no such effect, we do not regard this as established by our data. In somewhat stronger (0.0005 *M*) solutions of cyanide, the respiration of explants is reduced a little more for both dorsal (P barely over 0.01) and ventral ($P < 0.01$) explants:

(24) QO_2 (D,R) 0.0005 *M* KCN $= 0.10 \pm 0.02$ (2)

(25) QO_2 (D,L) control $= 0.26 \pm 0.04$ (2)

(26) QO_2 (V,R) 0.0005 *M* KCN $= 0.09 \pm 0.01$ (2)

(27) QO_2 (V,L) control $= 0.18 \pm 0.02$ (2)

But this effect is not a differential one ($P > 0.05$):

$$(28)\quad \frac{QO_2\ (D,R)\ KCN}{QO_2\ (V,R)\ KCN} = 1.1 \pm 0.1 \qquad (2)$$

$$(29)\quad \frac{QO_2\ (D,L)\ control}{QO_2\ (V,L)\ control} = 1.4 \pm 0.1 \qquad (2)$$

Summarizing: Although it is true that cyanide depresses the respiration of gastrula explants (as some function of its concentration), there is little indication from the respiratory effects that its action in blocking gastrulation is an anatomically localized one.

Respiration in sodium malonate (mal)

Another inhibitor of cellular oxidations is malonic acid which inhibits the conversion by succinic acid dehydrogenase of succinic acid to fumaric acid in the Krebs cycle. So far as we are aware, the only report of the effect of malonate on amphibian gastrula tissue is a report by Brachet and Rapkine (1939) to the effect that dorsal fragments develop normally in 0.025 *M* malonate. Precisely what this means is not clear.

But in new experiments, we have found that whole embryos placed at Stage 10 in 0.04 *M* sodium malonate do not develop past Stage 11^+. Furthermore, the respiratory activities of both dorsal and ventral explants are curtailed significantly ($P < 0.01$ in both cases) by malonate in this concentration:

$$(30)\quad QO_2\ (D,R)\ 0.04\ M\ mal = 0.13 \pm 0.01 \qquad (2)$$

$$(31)\quad QO_2\ (D,L)\ control = 0.32 \pm 0.03 \qquad (2)$$

$$(32)\quad QO_2\ (V,R)\ 0.04\ M\ mal = 0.11 \pm 0.01 \qquad (2)$$

$$(33)\quad QO_2\ (V,L)\ control = 0.26 \pm 0.03 \qquad (2)$$

But this curtailment is not a differential one ($P > 0.05$):

$$(34)\quad \frac{QO_2\ (D,R)\ mal}{QO_2\ (V,R)\ mal} = 1.2 \pm 0.1 \qquad (2)$$

$$(35)\quad \frac{QO_2\ (D,L)\ control}{QO_2\ (V,L)\ control} = 1.2 \pm 0.03 \qquad (2)$$

In summary: like other compounds which block dehydrogenase activities (cyanide, azide), malonate reduces the rate of oxygen utilization of gastrula tissues; but, again, the pattern of inhibition furnishes no clue (beyond some very general ones) to the manner in which malonate blocks gastrulation movements. We turn now to a different class of inhibitors.

Respiration in p-chloromercuribenzoic acid (HgB)

Barron and Singer (1945) and Singer and Barron (1945) have shown that p-chloromercuribenzoate is a powerful inhibitor of a large number of enzymes in-

volved in carbohydrate, protein, and fat metabolism. This reagent is thought to act by combining with all available —SH groups in native protein, unlike, for example, ferricyanide which acts only on those —SH groups close enough to form disulfides, or iodoacetate, which in low concentrations combines incompletely with even free —SH groups in native protein.

It is interesting that although 0.0001 *M* p-chloromercuribenzoate is able to prevent further development of gastrulae immersed in it at Stage 10, it has no respiratory effect whatsoever at this concentration ($P > 0.05$ in all cases):

(36) QO_2 (D,R) 0.0001 *M* HgB $= 0.31 \pm 0.06$ (2)

(37) QO_2 (D,L) control $= 0.36 \pm 0.08$ (2)

(38) QO_2 (V,R) 0.0001 *M* HgB $= 0.26 \pm 0.09$ (2)

(39) QO_2 (V,L) control $= 0.26 \pm 0.08$ (2)

(40) $$\frac{QO_2 \text{ (D,R) HgB}}{QO_2 \text{ (V,R) HgB}} = 1.3 \pm 0.2$$ (2)

(41) $$\frac{QO_2 \text{ (D,L) control}}{QO_2 \text{ (V,L) control}} = 1.4 \pm 0.1$$ (2)

Gastrulae placed at Stage 10 in 0.001 *M* p-chloromercuribenzoate do not develop further. But at *this* concentration the respiration of dorsal explants is sharply and significantly reduced ($P < 0.05$), and so is that of ventral explants ($P < 0.01$):

(42) QO_2 (D,R) 0.001 *M* HgB $= 0.10 \pm 0.02$ (2)

(43) QO_2 (D,L) control $= 0.28 \pm 0.05$ (2)

(44) QO_2 (V,R) 0.001 *M* HgB $= 0.08 \pm 0.01$ (2)

(45) QO_2 (V,L) control $= 0.23 \pm 0$ (2)

However, there is no differential effect ($P > 0.05$) of this —SH inhibitor on the respiration of dorsal and ventral explants:

(46) $$\frac{QO_2 \text{ (D,R) HgB}}{QO_2 \text{ (V,R) HgB}} = 1.2 \pm 0.1$$ (2)

(47) $$\frac{QO_2 \text{ (D,L) control}}{QO_2 \text{ (V,L) control}} = 1.2 \pm 0.2$$ (2)

Summarizing: p-chloromercuribenzoate will reduce the respiratory exchange of gastrula explants, but the effect upon dorsal and ventral explants is similar. Furthermore, it will block gastrulation in whole embryos. But the effect upon respiration and that upon gastrulation are not associated in any simple manner, for gastrulation may be completely blocked in the total absence of any effect upon the respiration.

Respiration in sodium fluoride (NaF)

Sodium fluoride is known to inhibit the conversion of 2-phosphoglyceric acid into phospho-enol-pyruvic acid by enolase (an enzyme requiring Mg ions) presumably by forming a complex magnesium fluorophosphate in the presence of inorganic phosphate (Baldwin, 1948)—thus preventing the anaerobic production of energy-rich phosphate bonds. In proper concentrations, it is well known to inhibit developmental processes. For example, developing amphibian embryos have been found to be checked by 0.02 *M* NaF but not by 0.005 *M* (Pomerat and Haringa, 1939; *R. pipiens*), and not by 0.01 *M* (Brachet, 1950; *R. fusca*). We have found that in 0.02 *M* NaF gastrulae immersed at Stage 10 do not develop past Stage 14; while in 0.04 *M* NaF they do not develop past Stage 11+. We have not investigated the effects of weaker concentrations.

But we have not confirmed Brachet's statement (1950, p. 327) that the respiration of amphibian embryos (*R. fusca*) is not fluoride-sensitive; for 0.02 *M* NaF significantly reduces the respiration of both dorsal ($P < 0.01$) and ventral ($P < 0.001$) explants:

(48) QO_2 (D,R) 0.02 *M* NaF $= 0.19 \pm 0.08$ (3)

(49) QO_2 (D,L) control $= 0.32 \pm 0.04$ (3)

(50) QO_2 (V,R) 0.02 *M* NaF $= 0.14 \pm 0.02$ (3)

(51) QO_2 (V,L) control $= 0.24 \pm 0.03$ (3)

As a result of this treatment, however, there was no significant change in the relative respiratory rates of dorsal and ventral explants ($P > 0.05$):

(52) $$\frac{QO_2 \text{ (D,R) NaF}}{QO_2 \text{ (V,R) NaF}} = 1.3 \pm 0.4 \qquad (3)$$

(53) $$\frac{QO_2 \text{ (D,L) control}}{QO_2 \text{ (V,L) control}} = 1.4 \pm 0.1 \qquad (3)$$

Summarizing: The pattern of fluoride inhibition of respiration of gastrula explants is much like that of other types of metabolic poisons: dorsal and ventral explants are similarly affected.

Respiration in 2,4 dinitrophenol (DNP)

We cannot confirm Andreassi's (1942) report (not available to us; see Brachet, 1950, p. 321) that dinitrophenol accelerates amphibian development; rather, our results support Dawson's (1938) results. Whole gastrulae placed at Stage 10 in 9.5×10^{-6} *M* 2,4 dinitrophenol (α-dinitrophenol; hereafter, DNP) do not develop past Stage 11. In spite of this, however, the tissues of such gastrulae respire at more than twice their normal rate. The respiration of dorsal explants is significantly increased ($P < 0.001$):

(54) QO_2 (D,R) 9.5×10^{-6} *M* DNP $= 0.66 \pm 0.21$ (4)

(55) QO_2 (D,L) control $= 0.31 \pm 0.09$ (4)

There is a similar significant increase in the respiration of ventral explants in DNP ($P < 0.001$):

(56) QO_2 (V,R) 9.5×10^{-6} *M* DNP $= 0.53 \pm 0.06$ (4)

(57) QO_2 (V,L) control $= 0.24 \pm 0.08$ (4)

But the relative rates of dorsal and ventral explants are not significantly different ($P > 0.05$):

(58) $\dfrac{QO_2 \text{ (D,R) DNP}}{QO_2 \text{ (V,R) DNP}} = 1.2 \pm 0.2$ (4)

(59) $\dfrac{QO_2 \text{ (D,L) control}}{QO_2 \text{ (V,L) control}} = 1.3 \pm 0.1$ (4)

In summary: this particular dinitrophenol behaves like other inhibitors of development in failing to act differentially upon the respiratory metabolism of dorsal and ventral explants. Its effect also provides another instance of the ease with which respiratory activity may be dissociated from morphogenetic activity. We now abandon the attempt to affect oxidative metabolism of dorsal and ventral explants differentially and turn to the question whether or not they differ in their anaerobic metabolism.

Anaerobic carbon dioxide production

Boell, Needham and Rogers (1939) reported that under anaerobiosis the CO_2-production of dorsal pieces of amphibian gastrulae is about three times greater than that of ventral pieces. Unfortunately, they believe also that there was an exactly analogous differential in anaerobic ammonia production, and their final values for CO_2-output were arrived at by taking into account the possibility that some 70% of the CO_2 produced was masked by ammonia, which combines with lactic acid produced anaerobically and hence prevents this acid from liberating CO_2 from the bicarbonate medium in which the explants were suspended. But we have recently (Gregg and Ornstein, 1952) tried to cast serious doubt on the reliability of their results: for in early gastrulae we were able to find no significant amount of ammonia produced by gastrula tissues—aerobically or anaerobically—and we have accounted for their ammonia values as artifacts. Consequently, we have felt it necessary to re-investigate the question of anaerobic CO_2-production by gastrula explants—and once again we have failed to confirm their findings.

Dorsal explants of gastrulae do, as they believed, produce CO_2 under anaerobic conditions:

(60) $Q_{an}CO_2$ (D,L and R) $= 0.11 \pm 0.04$ (14)

So do ventral explants, though at only a slightly lesser rate:

(61) $Q_{an}CO_2$ (V,L and R) $= 0.10 \pm 0.05$ (13)

This small difference between dorsal and ventral pieces, the ratio being:

$$(62)\quad \frac{\text{QanCO}_2\text{ (D,L and R)}}{\text{QanCO}_2\text{ (V,L and R)}} = 1.1 \pm 0.3 \qquad (13)$$

is not statistically significant ($P > 0.05$): thus we must conclude from these results that there is no striking difference between the anaerobic CO_2 output of these two parts of the amphibian gastrula.

Our results have been independently confirmed by Miss Adele Kostellow. With her kind permission her results (at 25° C.) are summarized below:

$$\text{QanCO}_2\text{ (D,L and R)} = 0.12 \pm 0.04 \qquad (8)$$

$$\text{QanCO}_2\text{ (V,L and R)} = 0.10 \pm 0.04 \qquad (8)$$

$$\frac{\text{QanCO}_2\text{ (D,L and R)}}{\text{QanCO}_2\text{ (V,L and R)}} = 1.4 \pm 0.7 \qquad (8)$$

As in our experiments, the difference between dorsal and ventral explants is not significant ($P > 0.05$). The high standard deviation (0.7) of her ratios is due to the presence of one experiment in which there was a ratio of 3. If this experiment is deleted, the standard deviation is reduced to 0.2, and the value of the ratio becomes 1.1.

It is not clear to us why our results are at variance with those of Boell *et al.* Perhaps their (apparently unwarranted) correction of CO_2-values by ammonia-values is partly to blame; but among other items which should be considered (and about which information is lacking) are: species-differences (they used *R. temporaria* and *Triton alpestris*); relative stages of gastrulae from which explants were made (we used Stage 10, they used more advanced stages, apparently); duration of anaerobiosis (about five hours for them, about two hours for us); and condition of the explants in the divers (never cytolyzed by interfaces in our experiments; apparently sometimes cytolyzed in theirs; *cf.*, their remarks, p. 353, and Brachet, 1950).

About the last point (explant injury) some comment should be made. In our experiments, the explants were suspended in Holtfreter's solution containing 0.03 *M* bicarbonate (pH after gassing: ca. 7.4), and in this solution the surface-coat of the explants tended to corrode slightly. Visual inspection revealed no apparent cytolysis, but as a confirmatory check on the normality of explants in this solution we obtained their oxygen uptakes, with the result that dorsal explants do indeed respire at a rate significantly different from that of dorsal explants in Holtfreter's without bicarbonate ($P < 0.001$; *cf.* (63) with (1) and (2)), while such ventral explants do not ($P > 0.05$; *cf.* (64) with (3) and (4)).

$$(63)\quad \text{QO}_2\text{ (D,L) } 0.03\ M\ \text{HCO}_3 = 0.27 \pm 0.07 \qquad (7)$$

$$(64)\quad \text{QO}_2\text{ (V,L) } 0.03\ M\ \text{HCO}_3 = 0.25 \pm 0.06 \qquad (7)$$

But, although the relative respiratory rate of dorsal and ventral tissues:

$$(65)\quad \frac{\text{QO}_2\text{ (D,L) HCO}_3}{\text{QO}_2\text{ (V,L) HCO}_3} = 1.1 \pm 0.2 \qquad (7)$$

is changed slightly by bicarbonate ($(P < 0.001)$; (65) compared with (5)), the change is so small that we do not consider it as indicating any crucial actual damage to the tissues.

Summarizing: Contrary to the report of Boell, Needham and Rogers (1939), dorsal and ventral explants do not produce carbon dioxide anaerobically at significantly different rates.

Summary

1. In full-strength Holtfreter's solution, dorsal explants from *Rana pipiens* gastrulae respire some 20% faster than ventral explants from the same gastrulae (with respect to dry weight). Lateral halves of dorsal explants respire at the same rate; and lateral halves of ventral explants respire at the same rate.

2. Azide, cyanide, malonate, p-chloromercuribenzoate, fluoride and 2,4 dinitrophenol in low concentrations all either completely block or greatly retard the gastrulation movements of *Rana pipiens* embryos.

3. Azide, cyanide, malonate, p-chloromercuribenzoate and fluoride all sharply reduce the respiratory rate of both dorsal and ventral explants from 29% (0.0001 *M* KCN) to 72% (0.001 *M* NaN_3).

4. 2,4 dinitrophenol (9.5×10^{-6} *M*) more than doubles the respiratory rate of both dorsal and ventral explants.

5. If these inhibitors block the movements of gastrulation by differential effects upon dorsal and ventral tissues, then these effects are not of such a nature as to be revealed by respiratory measurements—for the relative respiratory rates of dorsal and ventral tissues exposed to inhibitors are the same as those of controls in Holtfreter's solution.

6. Respiratory activity is not linked in any simple way with the movements of gastrulation, for compounds that reduce the former may have no effect upon the latter (0.0005 *M* KCN), and compounds that may completely block the latter have no effect upon the former (0.0001 *M* p-chloromercuribenzoate).

7. Dorsal and ventral explants of *Rana pipiens* gastrulae produce carbon dioxide anaerobically, but at rates that are not significantly different.

Literature Cited

Andreassi, G., 1942. Lo sviluppo delle uova e delle larve di Anfibi nelle soluzioni dilute di alfa-dinitro-fenolo. *Boll. Soc. Ital. Biol. Sper.*, **17**: 61–62.

Baldwin, E., 1948. Dynamic aspects of biochemistry. Macmillan Co., New York.

Barnes, M. R., 1944. The metabolism of the developing *Rana pipiens* as revealed by specific inhibitors. *J. Exp. Zool.*, **95**: 399–418.

Barron, E. S. G., and T. P. Singer, 1945. Studies on biological oxidations. XIX. Sulfhydryl enzymes in carbohydrate metabolism. *J. Biol. Chem.*, **157**: 221–240.

Boell, E. J., J. Needham and V. Rogers, 1939. Morphogenesis and metabolism: studies with the Cartesian diver ultramicrorespirometer. I. Anaerobic glycolysis of the regions of the amphibian gastrula. *Proc. Roy. Soc. London, Ser. B*, **127**: 322–356.

Brachet, J., 1950. Chemical embryology. Interscience Publishers, Inc., New York.

Brachet, J., and L. Rapkine, 1939. Oxydation et réduction d'explantats dorsaux et ventraux de gastrulas (Amphibiens). *C. R. Soc. Biol.*, **131**: 789–791.

Dawson, A. B., 1938. Effects of 2,4 dinitrophenol on the early development of the frog, *Rana pipiens*. *J. Exp. Zool.*, **78**: 101–116.

GREGG, J. R., AND N. ORNSTEIN, 1952. Anaerobic ammonia production by amphibian gastrulae explants. *Biol. Bull.*, **102**: 22–24.

HOLTER, H., 1943. Technique of the Cartesian diver. *C. R. Lab. Carlsberg, Sér. Chim.*, **24**: 399–478.

POMERAT, C. M., AND R. HARINGA, 1939. The effect of some inhibitors of carbohydrate metabolism on the development of the frog egg. *Anat. Rec.*, **75**, Suppl. I: 135.

SHUMWAY, W., 1940. Normal stages in the development of *Rana pipiens*. *Anat. Rec.*, **78**: 139–147.

SINGER, T. P., AND E. S. G. BARRON, 1945. Studies on biological oxidations. XX. Sulfhydryl enzymes in fat and protein metabolism. *J. Biol. Chem.*, **157**: 241–253.

SPIEGELMAN, S., AND F. MOOG, 1945. A comparison of the effects of cyanide and azide on the development of frogs' eggs. *Biol. Bull.*, **89**: 122–130.

SZE, L. C., 1952. Respiration of the parts of the *Rana pipiens* gastrula. *Physiol. Zool.* in press.

UMBREIT, W. W., R. H. BURRIS AND J. F. STAUFFER, 1945. Manometric techniques and related methods for the study of tissue metabolism. Burgess Publishing Company, Minneapolis.

THE EFFECTS OF CALCIUM ON ISOLATED ARTHROPOD MUSCLE FIBERS[1]

NATHANIEL K. PROCTOR[2]

Department of Zoology, University of Pennsylvania, Philadelphia 4, Pa., and Marine Biological Laboratory, Woods Hole, Massachusetts

The protoplasm of a muscle fiber is normally in a fluid state. This fluidity of muscle protoplasm has been demonstrated in frog muscle by Rieser (1949). Moreover, the protoplasm of muscle like that of other types of cells can and does undergo a gelation—similar to the clotting of blood. For a description of the clotting reaction see Speidel (1938); Heilbrunn and Wiercinski (1947); Woodward (1948). In marine eggs and protozoa, calcium and thrombin-like substances can cause protoplasm to clot (Heilbrunn and Daugherty, 1932). It is believed that the colloidal system of muscle is essentially similar to the colloidal system of marine eggs and protozoa and, in many respects, is similar to the colloidal system of blood. In an isolated cut muscle fiber the normally fluid protoplasm undergoes a gelation at the cut end of the fiber and this gelation progresses along the fiber (Heilbrunn, 1940). Here then is a system which can be studied. Woodward (1948) has studied the clotting properties of the vertebrate muscle. With few exceptions (for example, see Yaeger and Hager, 1934; Proctor, 1952) there is no available information concerning the effects of calcium on isolated invertebrate muscle fibers. The present study was performed on muscle fibers of invertebrates to determine: 1) whether or not the gelation of the muscle protoplasm is influenced by the calcium ion; 2) to compare the rate of clotting of invertebrate fibers with the rate of clotting of vertebrate fibers; and 3) to determine whether or not the clotting reaction can be inhibited.

The author wishes to express his appreciation to Dr. L. V. Heilbrunn of the Zoology Department, University of Pennsylvania, for his criticisms and kindness during the course of these experiments.

Materials and Methods

Various types of arthropods, both terrestrial and marine, were used in the present study of isolated single muscle fibers. The marine forms, which were obtained at the Marine Biological Laboratory (Woods Hole, Mass.), were *Callinectes sapidus* (blue crab) and *Homarus americanus* (lobster). The terrestrial arthropod used mainly in the experiments reported here was *Schistocerca americana* (grasshopper).

[1] Part of a thesis in Zoology presented to the Faculty of the Graduate School of the University of Pennsylvania in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

[2] Present address: Department of Biology, Morgan State College, Baltimore 12, Md.

Artificial Ca-free sea water served as the immersion medium for the marine arthropod studies. This modification of McClendon's artificial sea water was made up as follows: 490 cc. of 1 *M* NaCl, 10 cc. of 1 *M* KCl, 30 cc. of 1 *M* $MgCl_2 \cdot 6H_2O$, 20 cc. of 1 *M* $MgSO_4$ and distilled water to make a liter of solution. The pH of this solution varied from 7.8–8.6.

Bělař's (1929) insect Ringer, with calcium chloride omitted, was used as the dissecting medium for studies on the grasshopper.

Merck's reagent grade chemicals and glass (Pyrex) doubly distilled water were used exclusively in the preparation of all solutions.

All pH measurements were made with a glass electrode (Beckman) pH meter. Where necessary, pH adjustments were made with small quantities of 0.1 N NaOH or 0.1 N HCl.

A molar solution of calcium chloride was used as a stock solution and this was diluted with Ca-free Ringer to the desired concentration.

The femur of the large jumping leg (metathoracic) of the grasshopper was removed by cutting just distal to the coxotrochanter and just proximal to the femoral-tibial joint. The chitin was slit both along the anterior and the posterior part of the femur. This reveals the large plume-shaped muscles attached by their relatively large fibers to the chitin of the femur at one end and to a broad flat central tendon at the other. The chitin, which is now in two halves, can be removed by carefully pulling it in opposite directions from the distal end. This process leaves the entire muscle attached to one half of the cuticle. By grasping the muscle by its tendon at its distal end, the entire muscle may be peeled from the cuticle. This muscle appears relatively large, composed of numerous fibers varying in length (1.5–4.0 mm.) and diameter (0.15–0.29 mm.). The entire muscle was placed immediately in Bělař's (Ca-free) insect Ringer and single fibers were isolated by transverse cuts with steel dissecting needles, as close to the tendon as possible and cutting through the numerous tracheoles which bind the fibers.

The ends of the grasshopper muscle fibers, which are fastened to the chitinous exoskeleton, are covered with a brown or white pigment (depending upon the species). This pigmented end does not appear to be very permeable. Thus, the preparation essentially consists of a single muscle fiber each with one cut end.

After transferring a fiber to a depression slide with the aid of a pipette, the Ca-free insect Ringer was replaced with a test solution. A fresh cut was made across the fiber and the effects of the test solution were observed immediately. Some authors (see Woodward, 1948) rinse the fibers with two or three changes of the test solution. However, since all the fibers in these experiments were isolated in Ca-free insect Ringer, this technique was not thought necessary.

The clotted portion of the fiber was measured, at varying intervals (30 seconds–30 minutes) after cutting, under a magnification of approximately 43 ×, by means of a Ramsden-type filar micrometer eyepiece (Bausch and Lomb with 12.5 × eyepiece).

Muscle fibers of marine forms (crab and lobster) were isolated by cutting off the walking legs, removing the chitinous covering, and dissecting out single fibers from the exposed muscles in Ca-free sea water. In no cases were fibers used experimentally more than 45 minutes after amputation of the leg from the animal.

Results

Under the influence of calcium two visible changes occur in isolated single muscle fibers of arthropods: 1) a shortening of the fiber, and 2) the formation of a clot. Although these two phenomena occur concurrently in invertebrate muscle fibers, they will be considered separately in this paper.

1. Shortening

a. Blue crab. Single muscle fibers from the blue crab (*C. sapidus*), which had been isolated in Ca-free sea water, were placed immediately in isotonic calcium chloride solution (0.33 *M*). In each case, a fresh cut was made across the fiber and the length of the cut single fiber was then measured. As indicated by Tables I and II, these pieces of isolated muscle fiber varied in their original length. Following exposure to calcium the fibers shorten. The degree of shortening of each fiber was determined by the product of the final length times 100, divided by the

Table I

Effects of isotonic $CaCl_2$ (0.33 M) pH 5.7–6.0 (single muscle fibers)

Blue Crab (*Callinectes sapidus*)

Original length mm.	Time min.	Rate of clotting mm./min.	Final length mm.	% Shortened
3.519	1.0	0.247	1.711	51.3
2.608	1.0	0.219	1.556	59.6
3.651	1.0	0.293	2.934	19.7
3.995	1.0	0.472	1.980	50.5
3.504	1.0	0.446	1.801	48.7
3.463	1.0	0.352	2.078	40.0
3.569	1.0	0.802	2.475	30.4
3.341	1.0	0.207	1.548	53.7
3.260	1.0	0.393	1.483	54.5
5.623	1.0	0.262	2.863	48.6
3.260	1.0	0.328	2.773	14.0
2.950	0.75	0.958	2.078	29.6
3.039	1.0	0.413	1.752	42.4
3.260	1.0	0.195	2.387	26.8
2.542	0.5	1.03	1.418	44.3
2.852	1.0	0.250	1.344	52.9
2.811	0.25	0.995	1.956	30.5
2.991	1.0	0.351	1.589	49.6
3.121	1.0	0.487	2.094	33.0
2.469	1.0	0.387	1.548	37.3
3.285	1.0	0.359	2.135	34.1
4.792	1.0	0.328	1.996	58.4
3.537	1.0	0.767	2.942	16.9
		0.461		40.3

TABLE II

Effects of isotonic $CaCl_2$ (0.33 M) pH 8.0–8.2 (single muscle fibers)
Blue Crab (*Callinectes sapidus*)

Original length mm.	Time min.	Rate of clotting mm./min.	Final length mm.	% Shortened
4.360	1.0	0.588	1.654	62
3.243	1.0	—	1.670	48.5
5.321	1.0	0.608	1.956	63.3
5.599	1.0	0.328	3.553	36.6
5.028	1.0	0.467	3.088	38.6
3.349	1.0	0.640	2.326	31.2
8.581	1.0	0.452	4.360	49.2
3.594	0.5	0.502	1.793	50.2
7.359	1.0	0.502	2.591	64.8
7.726	1.0	0.410	5.028	35.0
8.655	1.0	0.443	5.868	32.3
6.414	1.0	0.561	3.742	41.7
6.715	2.0	0.294	4.319	35.7
		0.482		45.3

original length. This value when subtracted from 100 gives the per cent of shortening. This may be stated:

$$\text{per cent of shortening (p. s.)} = 100 - \left(\frac{\text{final length}}{\text{original length}} \times 100\right)$$

The shortening of fibers exposed to 0.33 *M* $CaCl_2$ (pH 5.7 to 6.0) varied from 14% to 59% (Table I). The average per cent shortening was about 40%. The pH of this solution (5.7–6.0) was lower than that of normal sea water. When isolated blue crab muscle fibers were exposed to 0.33 *M* $CaCl_2$ at the normal pH of sea water (8.0–8.2), the average shortening was 45.3% (Table II).

b. Lobster. Twelve fibers from the lobster (*H. americanus*), immersed in 0.33 *M* $CaCl_2$, showed an average shortening of 33.7%.

c. Grasshopper. The fibers of the grasshopper (*S. americana*) were extremely sensitive to calcium. Twenty-three fibers of this arthropod showed an average shortening of 58% in a solution containing calcium chloride in a concentration of 0.0018 *M*.

2. *Clotting*

The second visible change occurring in isolated single muscle fibers, as a result of the action of the calcium ion, is the formation of a clot. The clotted protoplasm appears only at the cut end or other point of injury of the muscle fiber. The area of the clot appears as a swollen region darker in color than the remaining protoplasm. This clot moves as a wave along the muscle fiber. In addition, the clotted portion is characterized by a loss of cross striations.

a. Rate of clotting.

1) Blue crab and lobster. Data were gathered at one-minute intervals to determine the rate of movement of the protoplasmic clot in isolated single muscle fibers of the blue crab under the influence of various concentrations of calcium. The effects of four different concentrations on blue crab (*C. sapidus*) are summarized in Figure 1. In all concentrations of calcium observed, there was a rela-

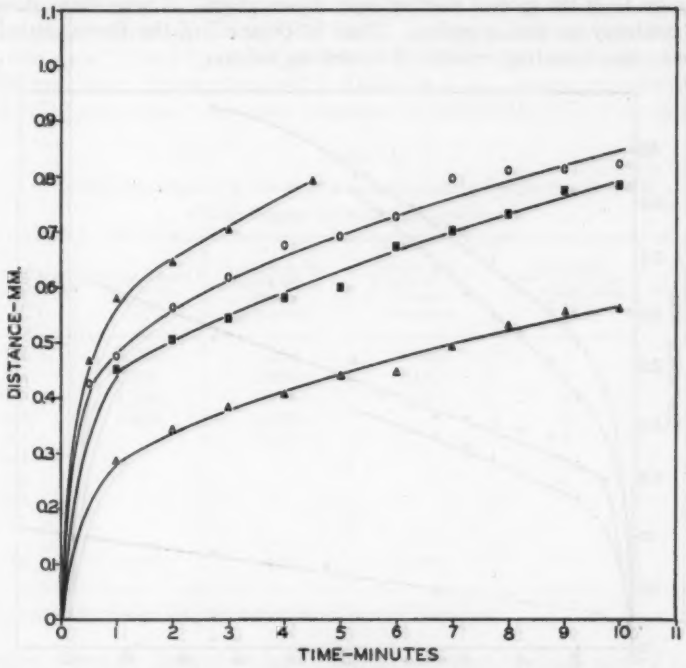


FIGURE 1. The effects of calcium on single muscle fibers of the blue crab (*Callinectes sapidus*). Legend: Open triangles (△), artificial sea water, pH 7.8, average of 10 fibers; solid squares (■), 0.083 *M* $CaCl_2$ in artificial Ca-free sea water, pH 8.2, average of 5 fibers; open circles (○), 0.165 *M* $CaCl_2$ in artificial Ca-free sea water, pH 7.5, average of 13 fibers; solid triangle (▲), 0.330 *M* $CaCl_2$ (pure), pH 6.89, average of 10 fibers.

tively high rate of movement of the protoplasmic clot of the muscle fibers during the first minute of exposure. However, this rate was not maintained at this high level for the entire period of exposure of these muscles in calcium. In Figure 1 the ordinates show the length of the clotted material in the fiber and the abscissae the time. The rates at which the clotted portion of the fiber increases in length are given by the slopes of the curves. In artificial sea water (0.0117 *M* $CaCl_2$), the average rate of movement of the protoplasmic clot of 10 fibers was 0.287 mm./min. at one minute and decreased to a rate of 0.108 mm./min. at 4 minutes (see Fig. 1). The average rate of movement of the protoplasmic clot of 5 fibers exposed

to 0.083 *M* $CaCl_2$ was 0.451 mm./min. at one minute and decreased to 0.075 mm./min. at 10 minutes. Thirteen fibers exposed to a concentration of 0.165 *M* $CaCl_2$ showed an average rate of clotting of 0.475 mm./min. at one minute and 0.0824 mm./min. at 10 minutes. Isotonic calcium chloride solution (0.33 *M*) gave a value (average of 10 fibers) of 0.534 mm./min. at one minute and 0.176 mm./min. at 4.5 minutes (see Fig. 1).

Attempts to obtain single muscle fibers from the lobster (*H. americanus*) suitable for a study of the rate of clotting were unsuccessful. These fibers showed an inherent tendency to stick together. This "stickiness" of the fibers caused them to adhere to the dissecting needles, thus causing injury.

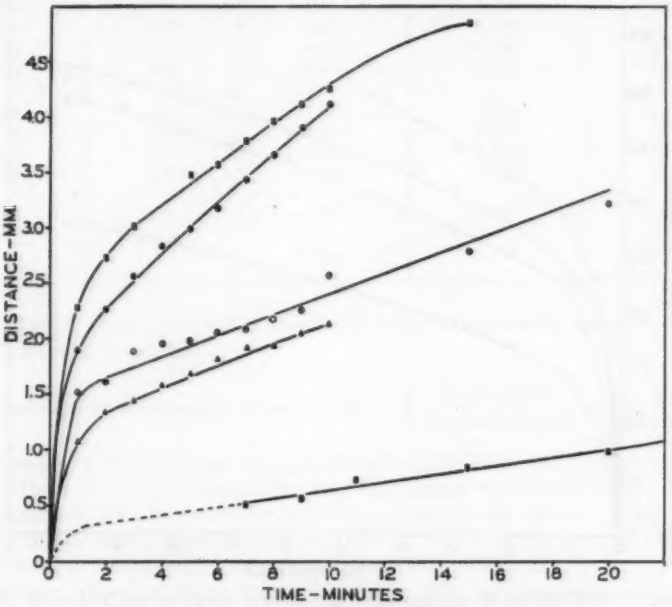


FIGURE 2. The effects of calcium on single muscle fibers of the grasshopper (*Schistocerca americana*). Legend: Open squares (□), 0.0175 *M* $CaCl_2$ in Ca-free Ringer, pH 7.25, average of three fibers; solid circles (●), 0.0087 *M* $CaCl_2$ in Ca-free ringer, pH 7.22, average of three fibers; open circles (○), 0.0044 *M* $CaCl_2$ in Ca-free Ringer, pH 7.32, average of 6 fibers; solid triangles (▲), 0.0018 *M* $CaCl_2$ in Ca-free Ringer, pH 7.5, average of 9 fibers; solid squares (■), Ca-free Ringer, pH 7.3, average of 5 fibers.

2) Grasshopper. Data were gathered at one-minute intervals to determine the rate of clotting of protoplasm in various concentrations of calcium. The wave-like movement of the clot along the muscle fiber showed considerable variation in speed even in fibers from the same animal.

Figure 2 summarizes the results of the effects of various concentrations of calcium on single muscle fibers of the grasshopper (*S. americana*). The ordinates show the length of the clotted material in the fiber, the abscissae the time. The

rates at which the clotted material increases in length are given by the slopes of the curves. Four concentrations of calcium chloride were used in these experiments (see Fig. 2) : 0.0175 *M* (top curve, open squares) ; 0.0087 *M* (solid circles) ; 0.0044 *M* (open circles) ; and 0.0018 *M* (solid triangles). The pH of these test solutions varied from 7.22–7.50. Muscle fibers exposed to these four test solutions show a relatively high rate of movement of the protoplasmic clot during the first minute of exposure. The effects of various concentrations of calcium on the rate of protoplasmic clotting of single muscle fibers during the first minute are as follows: fibers immersed in 0.0175 *M* $CaCl_2$ showed an average rate of clotting of 2.297 mm./min.; fibers immersed in 0.0087 *M* $CaCl_2$ had an average clotting rate of 1.899 mm./min.; fibers immersed in 0.0044 *M* $CaCl_2$ showed an average clotting rate of 1.512 mm./min.; and those immersed in 0.0018 *M* $CaCl_2$ (the calcium con-

TABLE III

Effects of calcium on rate of protoplasmic clotting (single muscle fibers)

Grasshopper (*Schistocerca americana*)

Ringer (.0018 *M* $CaCl_2$) mm./min.	Ca-free Ringer at 7 min. mm.	2.5 ×conc. Ca (.0044 *M*) in Ringer mm./min.	10 ×conc. Ca (.0175 *M*) in Ringer mm./min.	K oxalate (.005 *M*) in Ca-free Ringer after 15 min. mm.	Na oxalate (.005 *M*) in Ca-free Ringer after 15 min. mm.
1.281	0.536	1.616	2.315	0	0
1.144	0.526	1.498	2.143	0	0
1.053	1.135	1.798	2.433	0	0
0.962	0.490	1.398		0	0
1.262		1.253		0	0
0.944				0	0
0.726				0	0
1.088					
0.971					
1.050	0.671	1.512	2.297		

Figures show rate at one minute unless otherwise indicated.

centration normally present in insect Ringer) had an average rate of clotting of 1.050 mm./min. This relatively high rate of movement of the clot is not maintained during the later stages of the clotting process (see Fig. 2). Thus, the rate of clotting of fibers exposed to 0.0018 *M* $CaCl_2$ had an average clotting rate of 0.332 mm./min. at 5 minutes and 0.211 mm./min. at 10 minutes. Fibers exposed to 0.0044 *M* $CaCl_2$ showed an average rate of clotting of 0.394 mm./min. at 5 minutes and 0.234 mm./min. at 10 minutes. Those fibers immersed in 0.087 *M* $CaCl_2$ and those immersed in 0.0175 *M* $CaCl_2$ had an average rate of clotting of 0.596 mm./min. and 0.696 mm./min. at 5 minutes and 0.410 mm./min. and 0.425 mm./min. at 10 minutes, respectively.

The rate of movement of the protoplasmic clot of grasshopper muscle fibers in Ca-free Ringer is difficult to determine during the first few minutes of observation. These difficulties are encountered only during the first 6–7 minutes. This period is characterized by a gradual darkening of the protoplasm in the area adjacent to the cut or exposed end. There is no definite boundary separating the clotted re-

gion from the unclotted portion of the fiber. However, after 7 minutes, in all cases, there was a distinct clotted portion with a definite boundary characteristic of fibers exposed to higher concentrations of the calcium ion. In Figure 2 the time during which there is indistinct clotting is represented by a broken line. The average rate of clotting of 5 fibers at 7 minutes was 0.072 mm./min. and 0.066 mm./min. at 11 minutes.

b. Inhibition of clotting. It has been shown that the rate of movement of the protoplasmic clot in muscle fibers is dependent upon the calcium concentration. The question now arises as to whether or not the clot progresses in the absence of calcium.

1) Blue crab

With muscle fibers from the blue crab (*C. sapidus*), it was unnecessary to use sodium or potassium oxalate to prevent the movement of the protoplasmic clot. Fibers dissected and cut in Ca-free sea water and allowed to remain in this medium for periods of from 20–36 minutes showed no evidence of protoplasmic clotting

TABLE IV

Effects of calcium on rate of protoplasmic clotting (single muscle fibers)

Blue Crab (*Callinectes sapidus*)

Artificial sea water (0.0117 *M* $CaCl_2$) mm./min.	Isotonic $CaCl_2$ (.33 *M*) mm./min.	1/2 isotonic $CaCl_2$ (.165 *M*) mm./min.	Ca-free sea water (from 20–36 min.) mm./min.
0.287	0.586	0.448	0
0.362	0.513	0.456	0
0.382	0.578	0.619	0
0.225	0.448	0.464	0
0.327	0.741	0.619	0
0.265	0.448	0.448	0
0.205	0.432	0.456	0
	0.603	0.513	0
	0.472	0.554	0
0.293	0.534	0.508	

during this period of exposure (see Table IV). Upon return to artificial sea water containing a concentration of calcium of 0.11 *M*, these same fibers immediately showed the clotting reaction characteristic of muscle exposed to calcium.

2) Grasshopper

In the absence of calcium from the surrounding medium, the protoplasmic clot progresses. Thus, if a single muscle fiber from the grasshopper is isolated in Ca-free insect Ringer, a protoplasmic clot will form and progress (see bottom curve, Fig. 2). However, either sodium or potassium oxalate when mixed with Ca-free insect Ringer is effective in preventing the formation of a clot (see Table III).

The concentration of potassium (or sodium) oxalate which was most effective with grasshopper muscle fibers was 0.005 *M*. Higher concentrations usually resulted in death of the muscle fibers, whereas lower concentrations were not effective in preventing the movement of the clot. Figure 3 summarizes the results of experiments with potassium oxalate in inhibiting the rate of movement of the proto-

plasmic clot. A series of experiments was performed in which muscle fibers of the grasshopper (*S. americana*) were cut in a K-oxalate (0.005 *M*)-Ca-free insect Ringer solution and allowed to remain in this solution for 15 minutes. During this period, no protoplasmic clot could be observed in these fibers (see bottom curve, Fig. 3). However, after the 15-minute period had expired, each muscle fiber was placed in normal insect Ringer. There was an immediate clotting of the muscle protoplasm. The rate of movement of this protoplasmic clot was 1.245 mm./min. during the first minute and 2.040 mm./min. at the end of 5 minutes (average of 5

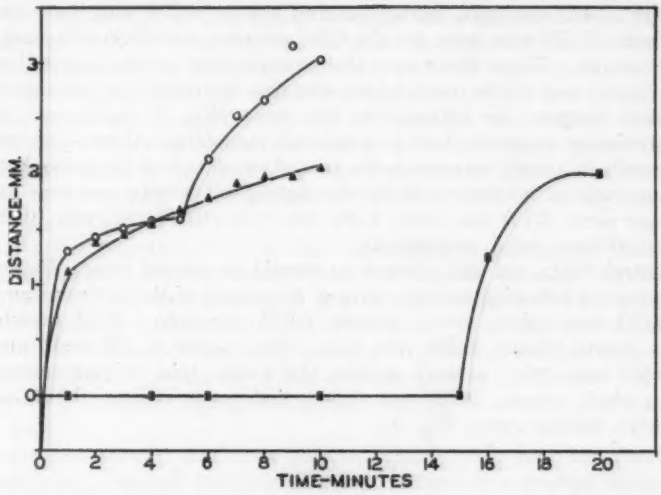


FIGURE 3. The effects of oxalate on protoplasmic clotting in single muscle fibers of the grasshopper (*Schistocerca americana*). Legend: Open circles (○), Ringer for one minute, K-oxalate (0.005 *M*) for the next 4 minutes, Ringer for 5 minutes, average of 6 fibers; triangles (▲), control, Ringer for 10 minutes, average of 5 fibers; squares (■), K-oxalate (0.005 *M*) for 15 minutes, Ringer for 5 minutes, average of 5 fibers.

fibers). These experiments clearly demonstrate the inhibitory effect of K-oxalate on the movement of the clot in muscle protoplasm. The effectiveness of both potassium and sodium oxalate is shown in Table III.

Another experiment was performed to determine whether this clotting reaction, once initiated by calcium, could be stopped. In this experiment a muscle fiber was first exposed to normal insect Ringer (containing calcium as the clotting agent) for a period of time sufficient to initiate the clotting reaction. Then the fiber was washed and the Ringer solution replaced with an oxalate-Ringer solution. After observations were made on the progress of the clot, the oxalate-Ringer solution was replaced with normal insect Ringer and the rate of clotting was again observed. The results of this experiment are shown in Figure 3 (top curve). A detailed account of this experiment follows. Muscle fibers were cut and allowed to remain in normal insect Ringer (calcium concentration, 0.0018 *M*) for one minute (see Fig. 3, top curve). During this period the average (6 fibers) rate of movement

of the clot was 1.300 mm./min. These fibers were then quickly washed three times with oxalate-Ringer (0.005 *M* K-oxalate in Ca-free insect Ringer) and allowed to remain in this solution for four minutes. The average rate of movement of the clot of these 6 fibers was determined at one-minute intervals during exposure to the oxalate-Ringer solution. In Figure 3 (top curve) the point at one minute indicates the distance the clot had progressed during a one-minute exposure to normal insect Ringer. The next four points (2, 3, 4 and 5 minutes) indicate the progress of the clot under the influence of oxalate-Ringer for 4 minutes. The average rates of movement of the protoplasmic clot while immersed in oxalate-Ringer were: 1.432 mm./min. during the first minute; 0.750 mm./min. during the second minute; 0.525 mm./min. for the third minute; and 0.936 mm./min. during the fourth minute. These fibers were then washed with normal insect Ringer and the rate of movement of the protoplasmic clot was determined at one minute intervals in insect Ringer. As indicated by the curve (Fig. 3, top curve), exposure of these previously oxalated fibers to a solution containing calcium (normal insect Ringer) results in a rapid increase in the rate of movement of the protoplasmic clot. The average rates of movement of the clot during a 5-minute exposure to normal insect Ringer were: 2.147 mm./min., 1.261 mm./min., 0.888 mm./min., 0.789 mm./min., and 0.606 mm./min., respectively.

Six control fibers, cut and allowed to remain in normal insect Ringer for 10 minutes, gave the following average rates of movement of the clot: during the first minute, 1.071 mm./min.; second minute, 0.673 mm./min.; third minute, 0.483 mm./min.; fourth minute, 0.396 mm./min.; fifth minute, 0.339 mm./min.; sixth minute, 0.302 mm./min.; seventh minute, 0.275 mm./min.; eighth minute, 0.243 mm./min.; ninth minute, 0.229 mm./min.; and tenth minute, 0.214 mm./min. (see triangles, middle curve, Fig. 3).

Discussion

Of the four major cations (Na^+, K^+, Mg^{++} and Ca^{++}) normally present in protoplasm, the calcium ion has long been known to be of primary importance in physiological processes. Its importance has been demonstrated in the clotting of blood and milk, the surface precipitation reaction of various cells, and more recently in relation to the stimulation in muscle. In fact, Heilbrunn has proposed a theory of stimulation and anesthesia based on the presence of calcium in the cortex of the cell and its subsequent movement to the interior. For a discussion of this theory and the relation of calcium to these phenomena see Heilbrunn (1943) and Woodward (1948). In order to study the effect of the calcium ion on muscle, physiologists have used isolated muscle fibers of vertebrates. The data in this paper reveal some of the similarities between vertebrate and invertebrate muscle fibers in their response to calcium.

One of the first visible effects of the calcium ion on vertebrate (frog) muscle fibers is a marked shortening of the fiber (Heilbrunn, 1940). In studies on isolated muscle fibers of three arthropods (blue crab, lobster and grasshopper) it was found that generally these forms follow the same pattern as vertebrate isolated muscle fibers. In the case of the lobster and the blue crab the fibers shortened to a lesser degree than isolated fibers from the frog in comparable solutions of calcium chloride. Thus, with frog fibers Heilbrunn found an average shortening of 73%.

The average percentage of shortening in muscle fibers of the blue crab was about 45% and in fibers of the lobster about 34%. Normal insect Ringer with a calcium concentration of 0.0018 *M* caused an average percentage of shortening of 58% in grasshopper muscle fibers. The evidence presented indicates that the sensitivity of muscle fibers of these forms to calcium is dependent upon the amount of calcium normally present in their environment.

A second visible effect of calcium on arthropod muscle fibers was the clotting reaction reported earlier by Speidel (1938), Heilbrunn and Wiercinski (1947) and Woodward (1948). The data show that this protoplasmic clotting in arthropod muscle is quite similar to that found by other investigators in vertebrate (frog) muscle fibers. However, the rate at which the movement of the protoplasmic clot occurs in the grasshopper indicates that the muscles of this animal are much more sensitive to low concentrations of calcium than those of the frog or the blue crab. Electrical stimulation has been used as a criterion for determining whether single muscle fibers of the frog are viable. With arthropod single muscle fibers, this method of determining their viability was found to be unreliable. A convenient indication of the viability of these fibers is the effect produced by the calcium ion—the clotting reaction.

It is obvious, therefore, that the effect of calcium on muscle protoplasm is much the same for muscle fibers of widely different types of animals. In frog muscle, however, it is difficult or impossible to prevent shortening and the clotting reaction by removing calcium from the surrounding medium. In all the invertebrate muscles I have studied, removal of calcium from the surrounding medium prevents shortening and clotting. This fact adds additional support to Heilbrunn's point of view, for it shows a complete dependence of this clotting of muscle protoplasm on the calcium ion.

Experiments showed that, once initiated, this clotting reaction, particularly in the grasshopper, does not stop immediately. There is a gradual decrease in the speed of movement of the clot in oxalate-Ringer solution. The data presented (Fig. 3) show a significant difference between the rate of movement of the clot in oxalate-Ringer solution as compared to normal insect Ringer. This suggests that not only is this clotting reaction a calcium-initiated phenomenon, but also that it requires calcium for its continuance.

Summary

1. When isolated single muscle fibers of blue crab, lobster and grasshopper have their cut ends exposed to solutions containing the calcium ion, they show the same effects as do frog muscle fibers; that is to say, they shorten and the protoplasm clots. However, the wave of clotting is faster in grasshopper muscle than it is in frog muscle. The degree of shortening of arthropod muscle fibers is less than that of frog muscle.

2. Evidence indicates that single muscle fibers of the grasshopper are more sensitive to calcium ions than are single fibers of the frog and blue crab.

3. The rate of movement of the protoplasmic clot in invertebrates is relatively rapid during the first minute. However, there is a gradual decrease in this rate after the first minute.

4. The clotting reaction can be prevented in invertebrate muscle fibers. In grasshopper muscle fibers, concentrations of 0.005 *M* sodium or potassium oxalate are effective in preventing the formation of the protoplasmic clot. Ca-free sea water is in itself sufficient to prevent the formation of the protoplasmic clot in muscle fibers of the blue crab. Once initiated, this clotting reaction in crab muscle cannot be stopped immediately by the application of sodium or potassium oxalate.

LITERATURE CITED

BĚLAŘ, K., 1929. Beiträge zur Kausalanalyse der Mitose. *Arch. f. Entw.*, **118**: 359–484 (after Romeis' Taschenbuch, p. 18).

HEILBRUNN, L. V., 1940. The action of calcium on muscle protoplasm. *Physiol. Zool.*, **13**: 88–94.

HEILBRUNN, L. V., AND K. DAUGHERTY, 1932. The action of sodium, potassium, calcium, magnesium ions on the plasmagel of *Amoeba proteus*. *Physiol. Zool.*, **5**: 254–274.

HEILBRUNN, L. V., AND F. WIERCINSKI, 1947. The action of various cations on muscle protoplasm. *J. Cell. Comp. Physiol.*, **29**: 15–32.

PROCTOR, N. K., 1952. The effects of various cations on insect muscle. *Physiol. Zool.*, **25**: 28–34.

RIESER, P., 1949. The protoplasmic viscosity of muscle. *Protoplasma*, **39**: 95–99.

SPEIDEL, C. C., 1938. Studies of living muscles. I. Growth, injury, and repair of striated muscle, as revealed by prolonged observations of individual fibers in living tadpoles. *Amer. J. Anat.*, **62**: 179–224.

WOODWARD, A. A., Jr., 1948. Protoplasmic clotting in isolated muscle fibers. *J. Cell. Comp. Physiol.*, **31**: 359–394.

YAEGER, J. F., AND ANNA HAGER, 1934. On the rates of contraction of the isolated heart and malpighian tube of the insect *Periplaneta orientalis:* Method. *Iowa State Coll. J. Sci.*, **8**: 391–395.

SEROLOGICAL COMPARISONS OF ASTACURAN CRUSTACEA[1]

CARLON W. PRYOR AND CHARLES A. LEONE

Department of Zoology, University of Kansas, Lawrence, Kansas

The methods of comparative serology have shown that the proteins of the sera of organisms are stable systems. These proteins are inherited in relatively simple ways and are not modified to any appreciable degree by environmental influences. Therefore such proteins can be considered in a natural scheme of classification. The purpose of the study here reported upon is to make serological comparisons of the serum proteins (the hemocyanins) of astacuran Crustacea. Eleven species from three families (Astacidae, Parastacidae and Homaridae) are studied.

Review of Literature

Earlier systematic serologists used various modifications of precipitin testing to establish relationships; only a few of the studies were quantitative. Within the past decade quantitative serological techniques have been developed to estimate the taxonomic relationships among animals.

Comparative serology of long-tailed Crustacea began with the studies of Nuttall (1904) and Graham-Smith (*in* Nuttall), both of whom used the same antiserum produced against the serum of *Homarus vulgaris* Milne-Edwards [= *Homarus gammarus* (Linnaeus)]. When reacted against a variety of other animal sera, the anti-*Homarus vulgaris* immune serum [= *H. gammarus*] reacted only with its homologous antigen and with the antigens of other decapods. As a result of these studies both Nuttall and Graham-Smith held that the degree of reaction between anti-*Homarus vulgaris* serum [= *H. gammarus*] and proteins of a variety of species was in general proportional to the amount of relationship of these proteins to each other. Their results, obtained from serological tests with animal bloods, did confirm, in general, the accepted classification of these animals which was based chiefly on comparative anatomy, embryology and paleontology.

Erhardt (1929), following some modifications of the methods used by Nuttall and by Graham-Smith, produced an antiserum against the serum of *Potamobius astacus* Linnaeus [= *Astacus astacus* (Linnaeus)], and reacted it against the homologous serum plus the sera of *Potamobius leptodactylus* Linnaeus [= *Astacus leptodactylus* Eschscholtz], *Homarus vulgaris* [= *Homarus gammarus*], several brachyuran species and numerous other invertebrates. The results of these experiments agreed comparatively well with the classification of these animals in that the sera of crayfish belonging to the family Astacidae reacted positively with the anti-*Potamobius astacus* serum [= *Astacus astacus*] whereas the sera of other decapods and schizopods gave negative results. Erhardt did not, however, discover the

[1] These studies were aided by a contract (NR 162-013) between the University of Kansas and the office of Naval Research, Department of the Navy.

value of employing antigens of known protein concentrations. The antigens studied in these experiments were arbitrarily diluted from 1:8 to 1:256 in a doubling-dilution series and tested with the anti-*Potamobius astacus* serum [= *Astacus astacus*]. Erhardt observed also that the antisera resulting from his injections of crustacean sera into rabbits were compounded of a series of partial precipitins which did not always develop in an equivalent manner in different rabbits exposed to the same antigen.

Clark and Burnet (1942) reported the separation of the various genera of Parastacidae from each other and from other Decapoda, by using the ring-test precipitin reaction, and absorption tests. Of the astacuran Crustacea, the hemocyanins of *Euastacus yarraensis* (McCoy), *Euastacus armatus* (von Martens), *Euastacus nobilis* (Dana), *Euastacus bispinosus* Clark, *Cherax albidus* Clark, *Cherax destructor* Clark, *Cherax rotundus* Clark, *Cherax bicarinatus* (Gray), *Cherax quinquecarinatus* (Gray), *Engaeus fultoni* Smith and Schuster and *Engaeus victoriensis* Smith and Schuster, were studied. The results obtained by Clark and Burnet indicated (1) the existence of several completely distinct "antigenic types" (*op. cit.*, p. 94) within the Decapoda, (2) the value of absorption experiments in studying close relationships, and (3) that hemocyanin was the only antigen in the serum of *Jasus lalandii* (Lamarck), a palinuran crustacean.

Boyden (1943) was the first to use a quantitative technique to obtain serological data for the determination of taxonomic relationships of long-tailed Crustacea. The degree of relationship between *Homarus americanus* Milne-Edwards and *Homarus vulgaris* Milne-Edwards [= *Homarus gammarus* (Linnaeus)] was determined by using a turbidimetric analysis of precipitates covering the whole range of reactivity between antigen and antiserum. Boyden concluded that the serological relationship of these species agreed with their generally accepted systematic positions, and that *Homarus americanus* and *Homarus vulgaris* [= *Homarus gammarus*] are valid species.

Leone (1950) reported further quantitative serological data from tests of some nephropsidean and astacuran Crustacea. Hemocyanins from *Homarus americanus* Milne-Edwards, *Homarus vulgaris* Milne-Edwards [= *Homarus gammarus* (Linnaeus)], *Cambarus clarkii* Girard [= *Procambarus clarkii* (Girard)], *Euastacus elongatus* Clark, *Euastacus nobilis* (Dana), *Euastacus armatus* (von Martens), *Cherax destructor* Clark and *Cherax albidus* Clark, were compared. The degree of relationship between the two species of *Homarus* agreed with the findings of Boyden in that they showed a high degree of serological correspondence. Considering only the serological data, Leone questioned whether the difference between them is sufficient to merit specific status for each. Leone concluded that *Euastacus elongatus, Euastacus nobilis* and *Euastacus armatus* exhibit a high degree of serological correspondence and are probably the same or closely related species. This conclusion confirms the findings of Clark and Burnet that *E. armatus* and *E. nobilis* are more closely related than either is to *E. sulcatus* or *E. bispinosus*. Leone further concluded that 20-minute reaction times for precipitin reactions established significant orders of relationships among animal sera. Twenty-four hour reaction time does not alter the order of relationship from that established at 20 minutes despite considerable increases in amounts of reaction recorded.

Leone and Pryor (1952) made serological comparisons of saline-hemocyanin filtrates of three species of shrimp, *Penaeus aztecus* Ives, *Penaeus setiferus* (Linnaeus) and *Penaeus duorarum* Burkenroad, using a photoelectric turbidimeter. These workers concluded that *P. aztecus* and *P. duorarum* are more closely related to each other than either is to *P. setiferus*. Their conclusion is in accord with Burkenroad's morphological arrangement of the three species (Burkenroad, 1949).

Materials and Methods

Antigens

Hemocyanin is the single protein in the sera of Crustacea (Allison and Cole, 1940; Clark and Burnet, 1942; Tyler and Scheer, 1945). This protein is an excellent antigen. The hemocyanins studied by us have been prepared with a minimum amount of preparatory treatment so that from physical handling, little or no chemical change occurred to alter biochemical characters. A list of the Crustacea from which the antigens were prepared is found in Table I. The taxonomic positions of species of the family Astacidae given in the Table follow those of Williams and Leonard (1952); the classification of species in the family Parastacidae is that of Hale (1927).

Kansas crayfishes

Approximately 50 specimens each of *Orconectes immunis* (Hagen), *Orconectes nais* (Faxon), *Orconectes neglectus* (Faxon) and *Procambarus simulans* (Faxon) were collected from ponds and streams in the state of Kansas and identified by the authors and by Dr. Austin B. Williams. As the crayfishes were received in the laboratory they were starved for 12 to 18 hours prior to bleeding, to remove the food materials that may have been present in the circulatory system and which might influence the true biochemical characteristics of the hemocyanins.

To obtain hemocyanins from the crayfishes they were bled by use of a 22 gauge needle and a 10 cc. syringe. A needle was inserted cephalad into the ventral abdominal artery and the blood drawn directly into the syringe. For each lot of crayfish of a given species, the bloods were pooled and placed in clean test tubes, allowed to clot, centrifuged at 2000 rpm for one-half to one hour, and the serum decanted. There remained after centrifugation a thick jelly-like clot which was wrapped in double thicknesses of fine cheese cloth and twisted manually to express the serum still remaining (Leone, 1949). These methods yielded a light bluish-green serum which was sterilized by filtering with Seitz filters, bottled in sterile five-cc. vials and stored at 0° ± 2° C., until needed for testing.

Orconectes virilis

Twenty-four specimens of *Orconectes virilis* (Hagen) were collected from Lake Winnebago, Wisconsin, and identified by Dr. A. Byron Leonard.

Hemocyanins from these crayfish were obtained by use of the procedure given for the Kansas crayfishes.

TABLE I

A list of species, the sera of which were used in the study of the relationships among astacuran Crustacea, and injection schedules of these sera used to produce antisera

Species	Serum protein gms./100 cc.	Injection schedule	Series of injections
Tribe Astacura			
Family Astacidae			
Procambarus simulans (Faxon)	2.50	0.25 cc., subcut., 0.1 cc., 0.2 cc., 0.5 cc., IV	1
Orconectes neglectus (Faxon)	3.00	0.25 cc., subcut., 0.1 cc., 0.2 cc., 0.5 cc., IV	1
Orconectes nais (Faxon)	8.00	0.25 cc., subcut., 0.1 cc., 0.2 cc., 0.5 cc., IV	1
Orconectes immunis (Hagen)	8.70	0.25 cc., subcut., 0.1 cc., 0.2 cc., 0.5 cc., IV	1
Orconectes virilis (Hagen)	5.65	0.25 cc., 0.5 cc., 0.75 cc., 1.0 cc., IV	2
Family Parastacidae			
Euastacus elongatus Clark	6.95	0.25 cc., 0.5 cc., 0.75 cc., 1.0 cc., IV	2
Euastacus nobilis (Dana)	3.90	0.25 cc., 0.5 cc., 0.75 cc., 1.0 cc., IV	2
Euastacus armatus (von Martens)	3.05	0.25 cc., 0.5 cc., 0.75 cc., 1.0 cc., IV	2
Cherax albidus Clark	5.40	0.25 cc., 0.5 cc., 0.75 cc., 1.0 cc., IV	2
Cherax destructor Clark	5.25	0.25 cc., 0.5 cc., 0.75 cc., 1.0 cc., IV	2
Family Homaridae			
Homarus americanus Milne-Edwards	3.35	0.5 cc., 0.5 cc., 0.5 cc., subcut.	3

Freshwater crayfishes from Australia

The sera from single specimens of *Cherax albidus* Clark, *Cherax destructor* Clark, *Euastacus armatus* (von Martens), *Euastacus elongatus* Clark, and *Euastacus nobilis* (Dana) were obtained from the collection of invertebrate sera at the University of Kansas. Originally these samples were prepared by Miss Ellen Clark, who collected the crayfishes from Broken Creek, Victoria, Australia. The same procedure as outlined for the Kansan crayfishes was used for the preparation of hemocyanins from the Australian species, with one exception: to each 9.0 cc. of serum was added 1.0 cc. of two per cent formalin solution which served as a bacteriostatic agent.

Homarus americanus

One of us (Leone) collected the hemocyanin from one specimen of *Homarus americanus* netted from the North Atlantic Ocean off the coast of Cape Cod, Massachusetts. A slit was made through the ventral hemocoel, at the junction of the cephalothorax and the abdomen, from which the animal was permitted to bleed through a clean, glass funnel into suitable containers. Whole blood obtained by this procedure was allowed to clot, and then centrifuged to hasten the ex-

pression of serum. "Merthiolate" in a dilution of 1 : 5,000 was added to the serum as a bacteriostatic agent.

Contamination of hemocyanin preparations

Some of the vials of hemocyanin preparations stored without preservatives became contaminated with bacteria despite the care exercised during filtration and bottling, but because of the stability of these proteins such contamination probably did not cause appreciable alteration. Leone (1949) observed that such contaminated hemocyanins stored over short periods at 0° ± 2° C. showed no detectable serological alterations after re-sterilization through Seitz filters.

Antisera

All antisera were produced in healthy, adult rabbits. A list of the antigens used to produce these antisera and their injection schedule is given in Table I. For the antisera to the proteins of Kansas crayfishes a pre-sensitizing dose of 0.25 cc. of antigen was injected subcutaneously, and the animal was permitted to rest for 30 days; each rabbit then received a single series of intravenous injections: 0.1 cc., 0.2 cc., and 0.5 cc., on alternate days. Rabbits producing the anti-*Homarus americanus* serum received three series of subcutaneous injections, each consisting of 0.5 cc., 0.5 cc., and 0.5 cc., on alternate days. The other antisera were produced by giving the rabbits two series of injections; a single series consisted of 0.25 cc., 0.5 cc., 0.75 cc., and 1.0 cc., given intravenously on alternate days.

Trial bleedings of the rabbits were made 8 days after the last injection of a series by taking whole blood from the median artery of the ear, while partial bleedings were made by direct cardiac puncture. For bleeding from the ear a 22 gauge hypodermic needle and a 10 cc. syringe were used. If, after either method of bleeding, there were sufficient antibodies in the serum for serological testing, a terminal bleeding was performed by direct cardiac puncture.

The whole blood obtained by the above methods was placed in clean test tubes and allowed to clot, after which it was rimmed, then placed at 0° ± 2° C. for 12 to 18 hours; the serum containing antibodies was then decanted. This antiserum was centrifuged to clear it of all blood cells, sterilized in Seitz filters, bottled in sterile vials, and stored at 0° ± 2° C. until used.

Methods of Testing

The methods of precipitin testing used in this study follow those outlined by Boyden and DeFalco (1943), and Leone (1949). Measurements of all turbidities were made with the Libby (1938) photronreflectometer. Critical analyses of the performance of the Libby photronreflectometer have been made (Boyden and DeFalco, 1943; Boyden, Bolton and Gemeroy, 1947; Bolton, Leone and Boyden, 1948). These authors conclude that for white precipitate systems, which include all the precipitin reactions, this instrument is valuable and reliable in studying the characteristics of such precipitates.

Precipitin tests were made by adding a constant amount of antiserum to doubling dilutions of antigens. The antigens were diluted with 0.9 per cent saline, buffered with $M/150$ phosphate salts (Evans, 1922). Tests were run in standard

Kolmer test-tube racks with a capacity of 48 tubes. A single test consisted of 17 Kolmer tubes into which was placed 1.7 cc. of diluted antigen; the first tube contained the initial dilution and each successive tube contained a protein dilution one-half the concentration of the preceding tube, ranging from 1:62.5 to 1:4,096,000. These dilutions were precisely made on the basis of the known protein concentration of the undiluted serum (Table I). Two control tubes were always used: a saline control, and an antiserum control which contained 1.7 cc. of the saline solution plus 0.3 cc. of antiserum. "Merthiolate" in a final dilution of 1:10,000 was added to prevent bacterial growth which would otherwise influence measurements of turbidities.

An antigen turbidity control value was obtained for each antigen dilution by transferring this solution to the cell of the photronreflectometer, and the amount of light reflected due to the cell itself plus its contents was then recorded and the dilution poured back into the Kolmer tubes. Readings for the antiserum and saline controls were likewise recorded. After the control readings for each dilution of antigen had been made, 0.3 cc. of antiserum was added and the tube placed in the dry air incubator maintained at 37° C. After 20 minutes, turbidities resulting from the antigen-antibody reactions were measured and recorded. Each tube was then stoppered with a cork to prevent excessive evaporation and left at room temperature for 24 hours, after which time readings were again made of the turbidities.

The control turbidities for the antigen and the antiserum were deducted from the total turbidity developed in any tube. The resultant values were assumed to be due to the precipitate formed through the inter-action of the antigen and antibodies in the system.

The range of pH for all reactants was between 7.0 and 7.2. Repeated precipitin tests were performed with an experimental error that did not exceed ± 2½ per cent.

Plotting of titration curves

Corrected values for antigen and antibody precipitates were plotted with the turbidities on the ordinate and the antigen dilutions plotted on the abscissa. The initial concentration of antigen was always placed at or near the intersection of the ordinate and abscissa. The homologous reaction (the reaction between an antiserum and the antigen used for its production) is the standard of reference for all heterologous reactions (the reaction between the same antiserum and the other antigens).

Values which are proportional to the areas of the curves are obtained by summating the plotted turbidity readings. These numerical values are statistical indices which serve to characterize curves for comparative purposes. The "areas" plus the plots of the curves provide analytical data on the biochemical nature of the hemocyanins which are being compared.

Preservatives in reactants

Marusich (1949) found that "Merthiolate" in the concentrations used in this study (1:5,000, and 1:10,000) did not alter the serological activity of hemocyanins

or the antisera produced against them. Leone (unpublished data) found that formalin at a concentration of 1 : 500 altered the serological activity of hemocyanins slightly but not enough to modify significantly the data which are presented in this paper.

Experimental Results

A summary of the serological relationships based on "areas" which were calculated from turbidity measurements taken at 20 minutes and 24 hours is presented in Table II. Plots of representative precipitin-curves are given in Figures 1 and 2.

Table II

Serological relationships among species belonging to the families Astacidae, Parastacidae and Homaridae from turbidity readings at 20 minutes and 24 hours

Antisera	Anti-*Orconectes neglectus* (Astacidae)		Anti-*Orconectes immunis* (Astacidae)		Anti-*Orconectes nais* (Astacidae)		Anti-*Orconectes virilis* (Astacidae)		Anti-*Procambarus simulans* (Astacidae)		Anti-*Cherax albidus* (Parastacidae)		Anti-*Cherax destructor* (Parastacidae)		Anti-*Euastacus nobilis* (Parastacidae)		Anti-*Homarus americanus* (Homaridae)	
Antigens																		
Orconectes neglectus	100	100	81	71	80	73			95	85								
Orconectes immunis	82	83	100	100	94	92	68	69	91	91	28	33	37	34	27	37	69	68
Orconectes nais	93	93	90	77	100	100	95	95	90	93	29	41	31	26	20	33	50	64
Orconectes virilis			73	68	81	81	100	100	93	96	33	37	26	26	23	35	50	64
Procambarus simulans			94	91	74	74			100	100	40	52	24	26	27	41	44	52
Cherax albidus	63	63	32	46	49	55	42	54	18	37	100	100	96	98	53	68	28	28
Cherax destructor	43	43	36	37	24	38	31	50	43	53	97	98	100	100	55	68	60	66
Euastacus elongatus					56	65	43	52	61	71	81	75	80	74			48	61
Euastacus nobilis	29	30	27	26	26	36			41	50	59	58	52	54	100	100	57	63
Euastacus armatus	16	22	30	34	25	35	38	43	44	54	65	69	53	54	90	92	55	64
Homarus americanus	17	21	31	37	26	32	33	38	5	14	11	26	1	6	19	25	100	100

Antisera produced with the small amounts of hemocyanins as given in Table I proved to contain sufficient quantities of precipitins to permit serological comparisons. Heterologous reactions did not in any case exceed homologous reactions. That the heterologous reactions did not exceed homologous reactions is in accord with the researches of Landsteiner (1945, p. 272), who found that antisera contain varying proportions of unique substances which will combine only with the homologous antigens. Heterologous antigens do not possess radicals identical to those of the homologous antigens, but may possess similar substances which allow reactions. Leone (1950, p. 124) and Boyden (1942) have defined this reaction as serological correspondence. Such correspondence when applied to antigens includes all antigens capable of reacting with an antiserum to any one of them, and when applied to antisera includes all antisera capable of reacting with any given antigen.

The precipitin curves proved to have single, narrow peaks, which strongly suggests that a single antigen-antibody system is involved in the reactions. In two instances, however, plots of turbidities revealed curves (Figures 1A and 1B) which were bimodal. Variations from a single modal curve have been considered as evidence of the probability of the presence of more than a single kind of antigen or

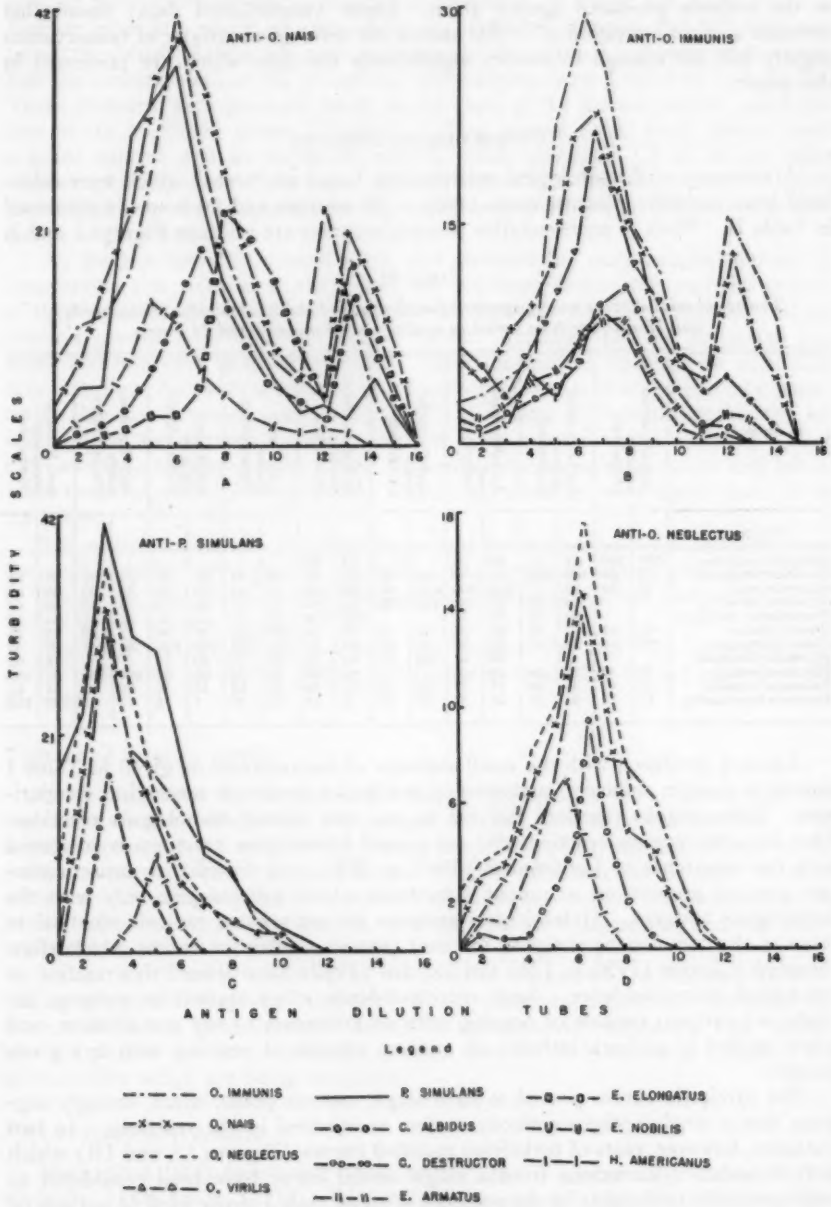

ANTI-O. NAIS
ANTI-O. IMMUNIS
ANTI-P. SIMULANS
ANTI-O. NEGLECTUS
TURBIDITY SCALES
ANTIGEN DILUTION TUBES
A
B
C
D
Legend
O. IMMUNIS
O. NAIS
O. NEGLECTUS
O. VIRILIS
P. SIMULANS
C. ALBIDUS
C. DESTRUCTOR
E. ARMATUS
E. ELONGATUS
E. NOBILIS
H. AMERICANUS

FIGURE 1

antibody in the system (Leone, 1949). Redfield (1934) concluded that hemocyanins are proteins composed of a prosthetic group consisting of a complex copper salt, of a sulfur compound, and a polypeptide and he indicated that several similar "units" may comprise the whole hemocyanin molecule. Since Svedberg and Pedersen (1940) by ultracentrifugation and sedimentation have been able to determine that hemocyanins have total molecular weights ranging from 300,000 to several million and are composed of polymeric fractions, it may well be that one or more of the groups of these molecular "units" may be responsible for secondary curves or variations from the unimodality of homologous curves.

The methods used in the collection and preparation of the hemocyanins have been kept as simple as possible; as a result little or no detectable chemical changes have occurred in the samples obtained. It is reasonable then to assume that they are representative biochemical entities for the species compared.

Twenty-minute reaction-time has been used extensively by Leone (1950), Boyden (1943), Leone and Pryor (1952) and others. A precipitin reaction may go to completion within 24 hours at room temperature. The binding of antibodies, as a rule, proceeds rapidly and in several experiments of Landsteiner (1945, p. 248) was found to be accomplished, for the most part, in less than a minute and never more than 15 minutes. This binding of antigen with antibody constitutes the first stage of the precipitin reaction. The formation of precipitates constitutes the second stage of antigen-antibody reactions and is a much slower process than is the combination of antigens with antibodies. The temperature at which the reaction takes place must be kept below that temperature which causes deterioration of the reactants. Increases in amounts of turbidities during a "prolonged" reaction-time have been recorded for this study. A homologous reaction, using anti-*Procambarus simulans* serum was read at 20 minutes, 40 minutes, 60 minutes and 24 hours. Increases in the homologous and heterologous reactions were proportional so that values obtained from turbidities after 20 minutes incubation at 37° C. were the same as those developed after 24 hours at room temperature. A homologous reaction using anti-*Orconectes nais* serum and a heterologous reaction using the protein of *Homarus americanus* were read at 20 minutes and again at 24 hours. These tests show increases in turbidities. At the region of optimal proportions higher peaks were obtained but with little or no shifting of these curves toward either the zones of antigen or antiserum excess.

Relationships

Serological tests involving species within the family Astacidae revealed that species within the genus *Orconectes* closely resemble one another and that the genus *Procambarus* is closely related but distinct from the genus *Orconectes* (Fig.

Figure 1. A and B. Bimodal curves from reactions of anti-*Orconectes nais* and anti-*Orconectes immunis* sera and the antigens with which they were tested. Reactions for *Orconectes* and *Procambarus* show high degrees of correspondence. Lesser degrees of correspondence are shown for *Cherax* and *Homarus*. C. Reactions of anti-*Procambarus simulans* serum against hemocyanins of closely related Crustacea belonging to the family Astacidae and other Crustacea belonging to the families Parastacidae and Homaridae. D. Reactions of anti-*Orconectes neglectus* serum with closely related Crustacea belonging to the family Astacidae and with species from the families Parastacidae and Homaridae.

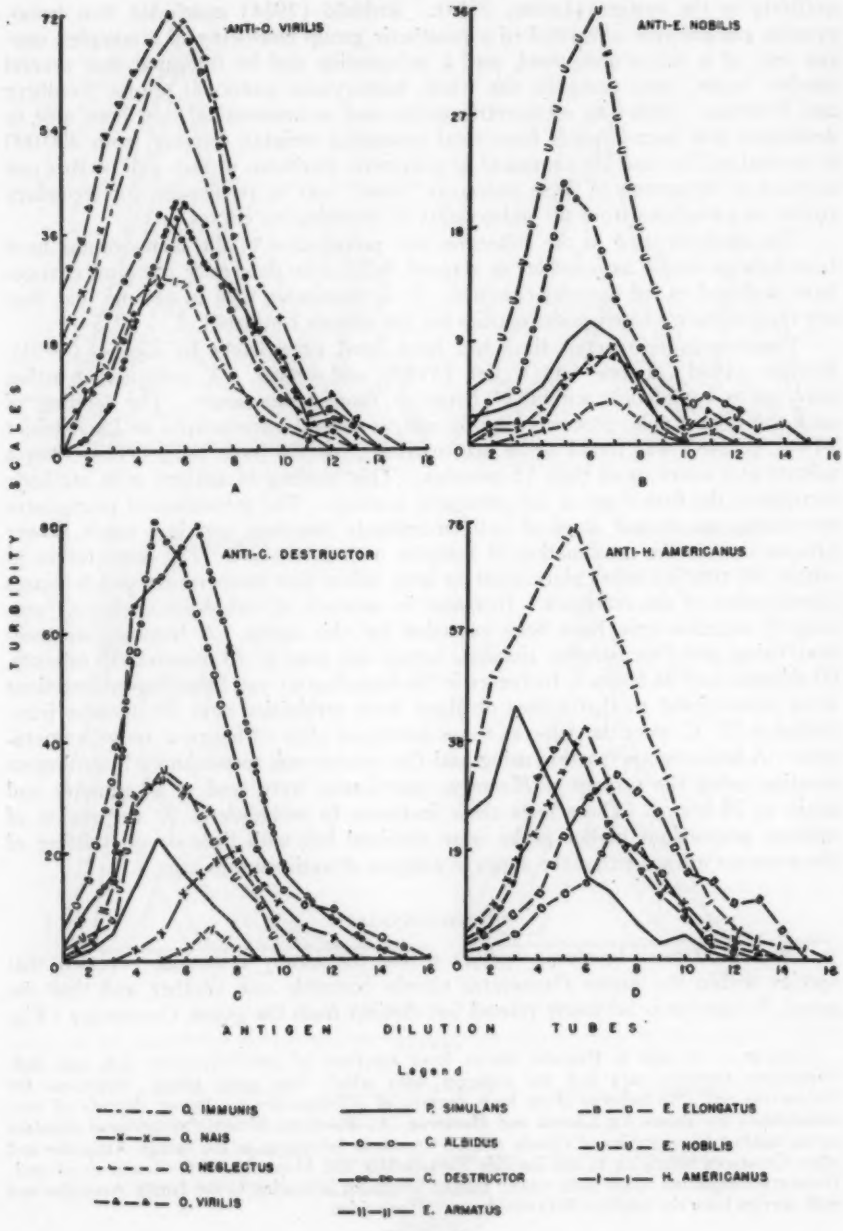

ANTI-O. VIRILIS
ANTI-E. NOBILIS
ANTI-C. DESTRUCTOR
ANTI-H. AMERICANUS
TURBIDITY SCALES
A
B
C
D
ANTIGEN DILUTION TUBES
Legend
O. IMMUNIS
O. NAIS
O. NEGLECTUS
O. VIRILIS
P. SIMULANS
C. ALBIDUS
C. DESTRUCTOR
E. ARMATUS
E. ELONGATUS
E. NOBILIS
H. AMERICANUS

FIGURE 2

1, Table II). An exception to expected relationships is indicated by anti-*O. immunis* serum and *P. simulans* protein (Table II). The hemocyanin of *P. simulans* when reacted with anti-*O. immunis* serum exceeds the reactivity of that of species belonging to the genus *Orconectes,* both at 20 minutes and 24 hours. Tests with all other antisera produced against species belonging to the genus *Orconectes* effectively separated *Procambarus* from the genus *Orconectes.* Tests with anti-*P. simulans* serum separated *Procambarus* and *Orconectes.* Some unknown circumstance in laboratory preparation may have altered the serological characteristic of the *Procambarus* proteins. Or, the *Procambarus* hemocyanin actually may possess more of the specific protein radical against which the antibodies were made than do the corresponding proteins of the species of *Orconectes.*

Our interpretation of the data (Table II) is that within the genus *Orconectes,* the species *O. immunis* and *O. nais* are more closely related to each other than to any other of the species tested. *Orconectes virilis* and *O. nais,* which have been considered to be the same species by some authors, appear to be distinct serologically. Both *O. virilis* and *O. neglectus* are more closely related to *O. nais* than to *O. immunis.*

On the basis of tests peformed with anti-Astacidae sera the hemocyanins from species of the families Parastacidae and Homaridae, in every instance, could be separated. The families Astacidae and Parastacidae resemble each other more than either does the family Homaridae. Tests with antisera produced against species of the family Parastacidae likewise separated hemocyanins of this family from those of the family Astacidae (Figs. 2B, 2C, 2D and Table II), and Homaridae. In this instance, also, the Homaridae is the more distant family.

An antiserum produced against the hemocyanins of *E. nobilis* proved effective in distinguishing among proteins of this species and those of species belonging to the families Astacidae and Parastacidae (Fig. 2B). To this antiserum the hemocyanins of species in the genus *Orconectes* and *Procambarus* reacted about equally.

Cherax albidus and *Cherax destructor* are closely related species (Fig. 2C, Table II). Antisera produced to the proteins of the two species closely resemble one another in their specificities. The antiserum against the hemocyanins of *Homarus americanus* gave remarkable differentiation between the two species of *Cherax.* This reaction may be anomalous.

Serological data indicate that *Euastacus armatus* and *E. nobilis* are more closely related than is *Euastacus elongatus* to either of them (Table II). From a study of these proteins, Clark and Burnet (1942) find that *E. armatus* and *E. nobilis* resemble each other more closely than either species does any other of the same genus. The results are also in accord with those obtained by Leone (1950) who found a high degree of serological correspondence among these three species of *Euastacus.*

FIGURE 2. A. Precipitin curves from reactions of anti-Astacidae serum with hemocyanins of species belonging to the families Astacidae, Parastacidae and Homaridae. High degrees of serological correspondence are shown for Parastacidae and Homaridae. B. Anti-Parastacidae serum showing degrees of relationship with hemocyanins from species belonging to the families Astacidae and Homaridae. C. Precipitin-reaction curves showing relationships among members of the families Parastacidae, Astacidae and Homaridae. D. Reactions of anti-*Homarus americanus* serum against hemocyanins of species from the genus *Orconectes, Procambarus, Cherax* and *Euastacus.*

Reactions with the antiserum to *Homarus americanus* indicate that this marine form is serologically distinct from the species in the two aquatic families. Moreover, tests with this antiserum (Fig. 2D, Table II) did not differentiate between Astacidae and Parastacidae. Leone (1950) reacted antisera produced against *Homarus americanus* and *Homarus vulgaris* [= *Homarus gammarus*] with hemocyanins of species belonging to both Astacidae and Parastacidae. He likewise was unable to differentiate these two families from the anti-Homaridae loci.

Discussion

Serological data are of value in constructing a natural system of classification, because (1) proteins are inherited traits which are not subjected to appreciable environmental influence, (2) comparisons can be made between corresponding proteins of the organism, and (3) chemical similarities and differences in antigens are revealed by adequate serological technique.

When corresponding proteins are to be differentiated quantitatively and qualitatively, antigens of known protein concentrations must be compared and the dilutions of each antigen must be equivalent. Antigens must be prepared so that little or no change in the chemical nature of these substances occurs.

In serological testing the rabbit is the greatest variable. Leone (1949, 1950) has shown that different rabbits receiving identical series of injections of the same antigen will produce antisera which differ considerably in their capacities to discriminate among antigens. From this it follows that serological comparisons based upon the production of one antiserum to any given antigen presents the possibility of obtaining only a minimum of data upon which serological comparisons may be based.

Summary and Conclusions

1. Serological comparisons among the hemocyanins of astacuran Crustacea have been determined using the precipitin test and a photoelectric turbidimeter. Five species of crayfish belonging to the family Astacidae (*Orconectes neglectus, Orconectes immunis, Orconectes nais, Orconectes virilis* and *Procambarus simulans*), five species of freshwater Australian crayfish belonging to the family Parastacidae (*Cherax albidus, Cherax destructor, Euastacus elongatus, Euastacus nobilis* and *Euastacus armatus*), and one species of Homaridae (*Homarus americanus*) were compared.

2. The species within the genus *Orconectes* are closely related. Serological data show that *Orconectes immunis* and *Orconectes nais* are most closely related to each other, and that *Orconectes neglectus* and *Orconectes virilis* are more closely related to *Orconectes nais* than to *Orconectes immunis.*

3. *Procambarus simulans* is closely related to species in the genus *Orconectes.*

4. The families Astacidae, Parastacidae and Homaridae are serologically distinct from one another. Tests with antisera produced against either the Astacidae or the Parastacidae have been able effectively to separate the other two families. On the other hand, tests with the anti-homarid serum were unable to separate differentially the families Astacidae and Parastacidae.

5. Within the family Parastacidae the genera *Cherax* and *Euastacus* are notably different serologically, but are more closely related to one another than to species from the Astacidae or Homaridae.

6. *Euastacus armatus* and *Euastacus nobilis* are more closely related to each other than either is to *Euastacus elongatus*.

7. *Homarus americanus*, in the family Homaridae, is distantly related to species belonging to the families Astacidae and Parastacidae.

8. Comparisons of relative relationships at 20-minute and 24-hour reaction times in serological relationships have been made.

LITERATURE CITED

ALLISON, J. B., AND WILLIAM H. COLE, 1940. The nitrogen, copper, and hemocyanin content of the sera of several arthropods. *J. Biol. Chem.*, **135**: 259–265.

BOLTON, ELLIS T., CHARLES A. LEONE AND ALAN A. BOYDEN, 1948. A critical analysis of the performance of the photronreflectometer in the measurement of serological and other turbid systems. *J. Immun.*, **58**: 169–181.

BOYDEN, ALAN A., 1942. Systemic serology: A critical appreciation. *Physiol. Zool.*, **15**: 109–145.

BOYDEN, ALAN A., 1943. Serology and animal systematics. *Amer. Nat.*, **77**: 1–22.

BOYDEN, ALAN A., AND RALPH J. DEFALCO, 1943. Report on the use of the photronreflectometer in serological comparisons. *Physiol. Zool.*, **16**: 229–241.

BOYDEN, ALAN A., E. T. BOLTON AND D. GEMEROY, 1947. Precipitin testing with special reference to the photoelectric measurement of turbidities. *J. Immun.*, **57**: 211–227.

BURKENROAD, MARTIN D., 1949. Occurrence and life histories of commercial shrimp. *Science* **110**: 688–689.

CLARK, ELLEN, AND F. M. BURNET, 1942. The application of serological methods to the study of the Crustacea. *Austral. J. Exp. Biol. Med.*, **20**: 89–95.

ERHARDT, ALBERT, 1929. Die Verwandschaftsbestimmungen mittels der Immunitatsreaktion in der Zoologie und ihr Wert für phylogenetische Untersuchungen. *Ergeb. und Fortschr. d. Zool.*, **70**: 290–291.

EVANS, A. C., 1922. A buffered physiological salt solution. *J. Infec. Dis.*, **30**: 95.

HALE, H. M., 1927. The crustaceans of South Australia. *Handbooks of the Flora and Fauna of South Australia, British Science Guild.*

LANDSTEINER, KARL, 1945. The specificity of serological reactions. Revised edition. Harvard University Press, Cambridge, Massachusetts.

LEONE, CHARLES A., 1949. Comparative serology of some brachyuran Crustacea and studies in hemocyanin correspondence. *Biol. Bull.*, **97**: 273–286.

LEONE, CHARLES A., 1950. Serological systematics of some palinuran and astacuran Crustacea. *Biol. Bull.*, **98**: 122–127.

LEONE, CHARLES A., AND CARLON W. PRYOR, 1952. Serological correspondence among three species of penaeid Crustacea. *J. Elisha Mitchell Scientific Society*, **68**: 27–31.

LIBBY, R. L., 1938. The photronreflectometer—an instrument for the measurement of turbid systems. *J. Immun.*, **34**: 71–73.

MARUSICH, WILBUR L., 1949. The effect of several preservatives on the turbidities developed in precipitin systems. Unpublished Master's thesis, Rutgers University, New Brunswick, New Jersey.

NUTTALL, G. H. F., 1904. Blood immunity and blood relationship. Precipitin tests. Cambridge, England, University Press.

REDFIELD, A. C., 1934. The hemocyanins. *Biol. Rev.*, **9**: 175–212.

SVEDBERG, T., AND K. O. PEDERSEN, 1940. The ultracentrifuge. Oxford at the Clarendon Press.

TYLER, A., AND B. T. SCHEER, 1945. Natural heteroagglutinins in the serum of the spiny lobster, *Panulirus interruptus*. II. Chemical and antigenic relation of blood proteins. *Biol. Bull.*, **89**: 193–200.

WILLIAMS, AUSTIN B., AND A. BYRON LEONARD, 1952. The crayfishes of Kansas. *Univ. Kansas Sci. Bull.*, **34**: 961–1012.

AN ANALYSIS OF THE AGGREGATION STAGE IN THE DEVELOPMENT OF THE SLIME MOLDS, DICTYOSTELIACEAE. II. AGGREGATIVE CENTER FORMATION BY MIXTURES OF DICTYOSTELIUM DISCOIDEUM WILD TYPE AND AGGREGATELESS VARIANTS[1]

M. SUSSMAN

Dept. of Biological Sciences, Northwestern University, Evanston, Illinois

In the development of the Dictyosteliaceae, aggregation begins when the population has reached the stationary growth phase. The individual myxamoebae, previously distributed at random, become elongated and radially oriented. They move in toward the centers of orientation and are crowded together in ramified streams as they do so. Mounds of cells are built up in this fashion and acquire the characteristic form of the pseudoplasmodium. Ultimately, the pseudoplasmodia give rise to organized, multicellular fruiting structures (Bonner, 1944).

Several features of this phenomenon have been investigated. Evidence obtained by Bonner (1947) supports the conclusion that aggregation is the result of a chemotactic response on the part of outlying cells to the production of a diffusible substance by individuals at or near the aggregative center. Study of interspecific and intergeneric cell mixtures (Raper, 1940) has indicated that not all species can enter communal aggregates and suggests that the aggregative response is specific.

The number of aggregative centers that can be formed by the myxamoebae is a function of both the population number and density (Sussman and Noël, 1952). Where the population density does not limit center formation, the number of centers produced is proportional to the number of cells. For *Dictyostelium discoideum* strain Nc-4, the number of centers/cell was found to be 4.73×10^{-4}, corresponding to a distribution of one center-initiating agency among approximately 2100 cells. For *D. purpureum* strain V-1, the value was 3.3×10^{-3}, equivalent to one center-forming agency among 300 cells. Examination of the distribution of center-forming capacity among small, replicate population samples revealed that under the conditions employed only a small proportion of the population possesses the ability to initiate the aggregative process.

One pertinent question, which could not be answered by study of the wild type populations exclusively, is concerned with whether or not single cells act as initiators of aggregation. The reasons for this limitation are given in another part of this paper. In order to settle the question, an analysis has been made of aggregations carried out by mixtures of wild type myxamoebae and aggregateless variants which are capable of responding to the aggregative stimulus but not of producing it. The results demonstrate the single cells do indeed initiate aggregation. Several other aspects of these mixed aggregations have been investigated and are described.

[1] This investigation was supported by a grant from the Division of Research Grants and Fellowships, U. S. Public Health Service.

Methods

A. Organisms and growth conditions

The strains used in this investigation were: *Dictyostelium discoideum* strain Nc-4 and two aggregateless variants of this stock, Nc-4 agg-53 and Nc-4 agg-53A. These were routinely grown in association with *Aerobacter aerogenes* on an agar medium whose composition has been given elsewhere (Sussman, 1951).

The technique of clonal plating, used in certain of the experiments reported here, has been previously described (Sussman, 1951). It is similar to the "spread plate" technique employed in the past for bacteriophage (Delbrück, 1946). The plaques of myxamoebae appear within the layer of bacterial growth after 3.5 days incubation at 22° C. During the following 24 hours, fruiting structures appear in the plaques.

B. Preparation of cells for aggregation experiments

44- to 50-hour plates containing myxamoebae in the stationary growth phase were harvested with cold, distilled water. The suspensions were spun in an International refrigerated centrifuge for 5 minutes at 1000 rpm in order to separate the myxamoebae from the remaining bacteria. After three more washes, the cells were suspended in Bonner's salt solution (Bonner, 1947). At this time, direct cell counts of the suspensions were made in the Levy chamber on 6 to 8 replicates. The variances encountered were satisfactorily small.

C. Deposition of cells on aggregation plates

The aggregation experiments were carried out on a washed agar-distilled water substratum, hereafter denoted as "minimal agar." This was prepared by washing 25 g. of Difco agar in 6 liters distilled water on a Buchner table-top filter. After washing, the agar was suspended in one liter of water and autoclaved. Plates were poured from this medium.

When aliquots of cell suspensions, ranging in volume from 0.01 to 0.2 cc., are placed on minimal agar, the cells are distributed homogeneously over a surface area whose size is a function of the volume delivered. In this manner, both the population number and density may be controlled effectively and the myxamoebae are found to aggregate and produce normal fruiting bodies.

Experiments to be described indicate that when cells are prepared by the procedure given and are deposited on minimal agar, no appreciable increase in cell number occurs after as much as 48 hours incubation. It must be noted, however, that the separation of myxamoebae from occluded bacteria is occasionally unsatisfactory. With such preparations, no trouble is encountered where the population on the minimal agar plates is very dense since the amoebae ingest the bacteria almost immediately. But in aliquots where the population density is not great, some bacteria escape ingestion long enough to form microcolonies. When these are finally entered by the myxamoebae, substantial proliferation ensues. This difficulty can be avoided almost completely by careful washing of cells and of the agar used in the minimal medium.

Results

A. Descriptions of the aggregateless variants

Two variants were employed in this investigation. The first of these, Nc-4 agg-53, was obtained by irradiation of the wild type with UV. The techniques used for its isolation as well as for the collection of a series of stocks displaying a wide spectrum of aberrant developmental patterns will be described elsewhere (Sussman and Sussman, in preparation).

The plaques produced by agg-53 are easily distinguishable from those of the wild type. The latter forms a spreading plaque with an irregular outline and diffuse periphery. Normal fruiting structures appear within its confines. In contrast, the plaque formed by agg-53 is much smaller, circular and has a very sharply defined periphery. A circular slime deposition or series of concentric depositions always appears, giving each plaque the appearance of a bull's eye. No signs of aggregation or the erection of fruiting structures are observable. Spores are not produced.

During serial passage of agg-53, a plaque type variant was found to arise occasionally. Upon sub-culture, it bred true and was designated Nc-4 agg-53A. The plaque produced by this variant spreads and has an irregular outline like that of the wild type. The interior of the plaque remains perfectly smooth. No signs of aggregation or the erection of fruiting structures are seen. Spores are not formed.

In the course of many serial passages, neither of the variants has been seen to produce a reverted or mosaic clone. However, this extreme stability is by no means true of all aggregateless stocks thus far isolated.

The question of whether or not the strains represent genic modifications cannot be answered since, at present, no system of recombination analysis is available for use. Arguments not based on segregation data are neither necessary nor sufficient for the distinction to be drawn and therefore will not be considered at this time.

It should be noted that Pfützner-Eckert (1950) has reported the isolation of a strain of *D. mucoroides* which is incapable of forming fruiting structures. From this author's description, however, it appears that the cells can perform some of the activities associated with aggregation and, under some conditions, even form small pseudoplasmodia. We have isolated similar varieties. Such cells when washed and distributed on minimal agar generally are capable of aggregating and producing normal mature fruiting structures although usually not in as great a number as the wild type produces. The inability of such cells to do the same on the growth plates is a problem which has not as yet been attacked.

B. The formation of aggregative centers by mixtures of Nc-4 wild type and the variants

A previous investigation (Sussman and Noël, 1952) has demonstrated that the number of aggregative centers produced by Nc-4 wild type cells is a function not only of the number of cells present but also of the population density. When the latter is less than approximately 100 cells/mm.^{2}, no centers are formed. As the density is raised (while the number of individuals is kept constant), the number of centers increases until at a density of about 200 cells/mm.^{2} a maximal number of centers is produced. This circumstance made it possible to determine if the ag-

gregateless individuals could respond to the stimulus imposed by wild type where the population density of the wild type was so low that, alone, they could not form centers.

Suspensions of washed Nc-4 wild type and agg-53, prepared according to the procedure previously described, were counted in the Levy hemocytometer and appropriate dilutions were made. From these, mixtures were arranged such that they contained a constant number of Nc-4 cells but varying numbers of aggregateless individuals. For example, in one experiment the mixtures all contained 5.0×10^5 cells/cc. of wild type while the numbers of agg-53 cells/cc. ranged from 5.0×10^6 to 6.0×10^7. Replicate drops of 0.01 cc. volume were distributed on

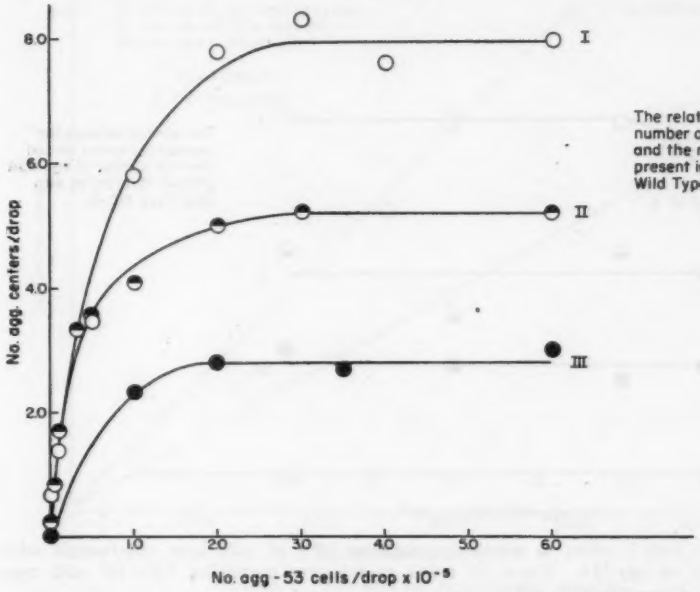


FIGURE 1. Curve I refers to mixtures containing 8.0×10^5 wild type cells/sample with various numbers of agg-53 cells. Curve II refers to mixtures containing 5.0×10^5 wild type/sample and Curve III, 2.6×10^5.

minimal agar, 10 drops to a plate and two or three plates for each mixture. Thus, the number of Nc-4/drop was 5.0×10^3 while the numbers of agg-53/drop varied between 5×10^3 and 6.0×10^5. Drops were also dispensed from control suspensions containing either Nc-4 or agg-53 in concentrations comparable to those in the experimental suspensions.

After distribution of the drops, the plates were allowed to stand with covers ajar until the drops had dried. The plates were incubated at 22° C. and twice during the following 48 hours the drops were examined either under 20 × or 100 × magnification in order to count the aggregates or fruiting bodies derived therefrom.

Thirteen of these determinations were made using seven different quantities of Nc-4 cells/drop ranging from 1.0×10^3 to 1.0×10^4. Figure 1 shows the results of three such experiments. Each point in the curves represents the mean of 30 replicate drops.

In the absence of agg-53 cells, 2.6×10^3 Nc-4 produced no centers (curve III of Fig. 1), as a result of the extremely low population density. (From the average radius of the drops and the number of cells deposited, the density was estimated to be less than 50 cells/mm.^{2}) Alone, 5.0×10^3 Nc-4 formed a mean of 0.21 centers/drop; 8.0×10^3 Nc-4 produced 0.7 centers/drop. Control suspensions of agg-53 cells were never observed to give rise to aggregate centers. Addition of

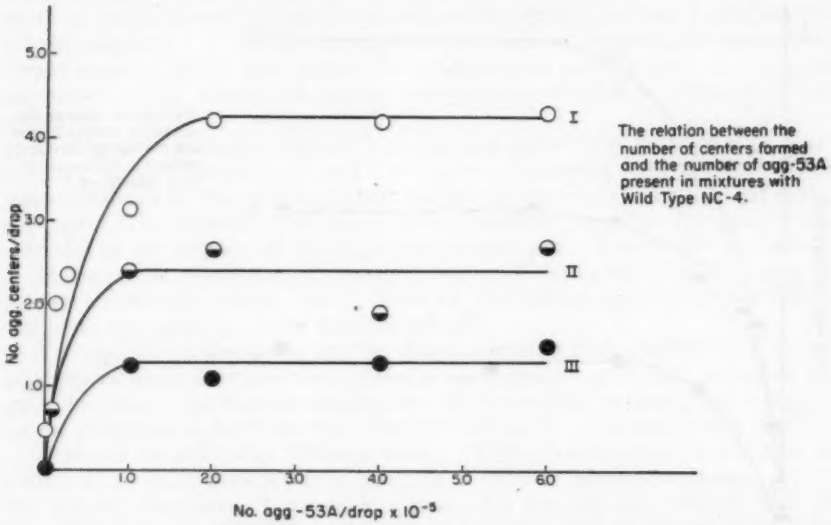


FIGURE 2. Curve I refers to mixtures containing 8.0×10^3 wild type cells/sample with various numbers of agg-53A. Curve II refers to mixtures containing 5.0×10^3 wild type cells/sample and Curve III, 2.0×10^3.

agg-53 cells to the wild type suspensions, however, markedly increased the numbers of centers formed. The presence of between 2 and 3×10^5 agg-53 cells resulted in maximal center formation whose absolute value depended upon the number of wild type. Thus for 8×10^3 Nc-4, a plateau value of 7.9 centers/drop was reached. For 5×10^3 Nc-4 the value was 4.9 and for 2.6×10^3 Nc-4 it was 2.8. One center was produced for approximately 1000 wild type cells present.

A similar set of experiments was performed using mixtures of Nc-4 and agg-53A. Figure 2 summarizes the results of three determinations. The curves are in qualitative agreement with those of Figure 1 except that significantly different plateaus were reached for the same numbers of Nc-4 employed. Using agg-53A as the test system, one center was formed for approximately every 1700 Nc-4 added.

C. The number of cells required to initiate the formation of an aggregative center

It has been mentioned that the results of a previous investigation indicate that only a small proportion of the myxamoeboid population possesses the ability to initiate center formation under the conditions of the experiment. The question of how many cells were required to initiate a center could not be answered conclusively from the data although a number of arguments could be raised to support the interpretation that a single cell can evoke the aggregative process. The inconclusiveness proceeded from the fact that in a population of wild type cells, one is concerned not only with the ability of a small proportion to initiate aggregation but also with

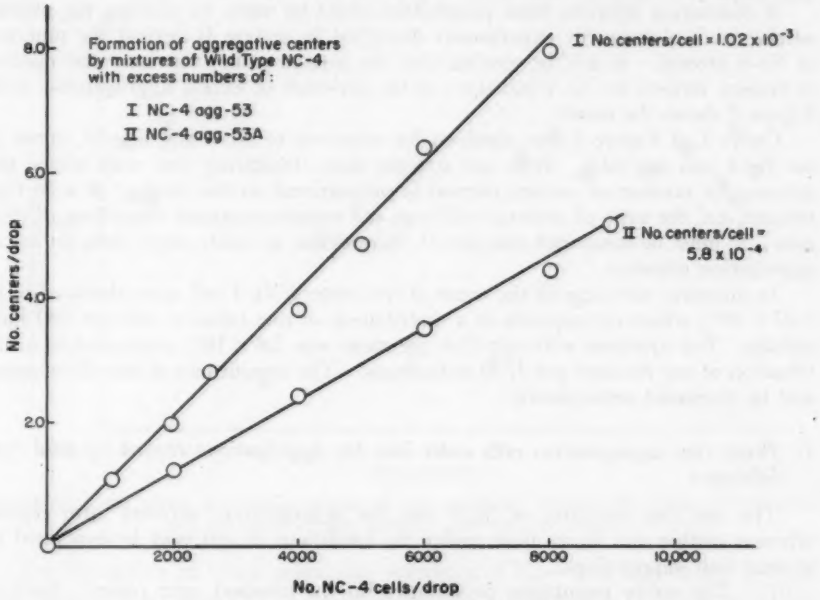


FIGURE 3. See text for experimental details.

the ability of the remaining individuals to respond to the initiating stimulus. From examination of the dependence of center formation on population number and density, it became clear that both abilities played decisive roles in the process. Thus, one could not, with this system, hope to dissociate the one from the other.

The use of aggregateless variants as the test system was considered to be capable of achieving the desired dissociation since with this system it became possible to alter the number of responding cells to any extent without changing the number of wild type individuals and therefore the number of initiator cells. Conversely, the presence of excess aggregateless cells would ensure that neither the number nor density of responding cells would limit the formation of centers. In this event, alteration of the number of wild type cells could only affect the number of initiators and it became possible therefore to examine initiating ability, *per se*.

Where the responding cells could not limit center formation, the initiation of centers by single cells would be evidenced by the fact that the number of centers formed would be directly proportional to the number of initiator cells present. For mixtures of Nc-4 and excess aggregateless variants, this would mean that the number of centers would, in fact, be proportional to the number of wild type, *i.e.,* the ratio of number of centers/wild type cell would remain constant regardless of the absolute number of wild type present. If, however, two or more cells must collaborate to evoke aggregation, the number of centers would in general vary exponentially with the number of wild type present and the ratio, number of centers/wild type cell, would approach the limit zero at infinite dilution.

A distinction between these possibilities could be made by plotting the plateau values obtained from the experiments described in section B against the numbers of Nc-4 present. It will be recalled that the plateau values represent the number of centers formed by Nc-4 initiators in the presence of excess aggregateless cells. Figure 3 shows the results.

Curve I of Figure 3 was obtained for mixtures of Nc-4 and agg-53, curve II for Nc-4 and agg-53A. Both are straight lines, indicating that with either test system, the number of centers formed is proportional to the number of wild type present, *i.e.,* the ratio of centers/wild type cell remains constant regardless of dilution. It must be concluded that for *D. discoideum,* at least, single cells do act as aggregation initiators.

In mixtures with agg-53 the mean of the centers/Nc-4 cell was calculated to be 1.02×10^{-3}, which corresponds to a distribution of one initiator cell per 980 individuals. For mixtures with agg-53A the mean was 5.8×10^{-4}, equivalent to a distribution of one initiator per 1720 individuals. The significance of this discrepancy will be discussed subsequently.

D. Proof that aggregateless cells enter into the aggregations evoked by wild type initiators

The fact that mixtures of Nc-4 and the aggregateless variants form centers whereas neither can do so alone under the conditions of test may be explained by at least four suppositions.

(a) The entire population proliferates on the minimal agar plates. Each of the cells originally laid down therefore produces a microclone. The Nc-4 cells increase in number to the extent that some can aggregate without assistance from the aggregateless individuals.

(b) The total population remains relatively constant on the minimal agar. However, the wild type cells increase in number at the expense of the aggregateless individuals. This increase might be due to growth or conceivably to the transformation of aggregateless variants into wild type. In either case the proportion of Nc-4 in the population would rise. The net increase would be sufficient for them to aggregate without assistance from the agg-53 cells.

(c) The numbers of both Nc-4 and agg-53 cells remain constant. The aggregateless cells act as "conductors" of the stimulus imposed by initiator cells in the wild type population. This conductance enables the responding cells of the wild type population to aggregate without, however, the active participation of the aggregateless individuals in the aggregation.

(d) The aggregateless cells, like the responding cells of the wild type population, are affected by the initiating stimulus and enter into the aggregate eventually formed.

It was possible to test the validity of the first two suppositions by making differential and total counts of the mixed population on the minimal agar plates at intervals between the time of deposition and the onset of aggregation. Were assumption (a) valid, the total population would increase significantly. Were assumption (b) valid, the proportion of Nc-4 in the population would increase significantly.

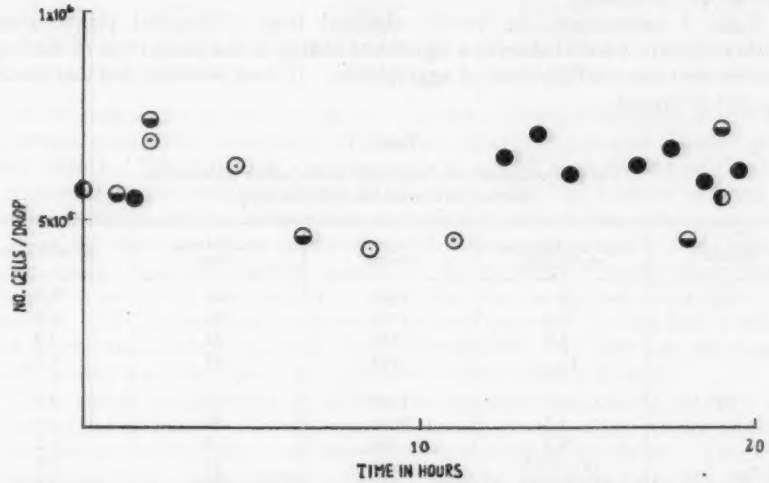


FIGURE 4. Total viable population counts of cell mixtures between the time of deposition and the onset of aggregation. See text for details.

The deposition of drops containing mixtures of Nc-4 and agg-53 on minimal agar plates was accomplished in the usual manner. Two mixtures were used: 10% Nc-4 cells (I) and 1.5% Nc-4 (II). The latter value falls within the limits employed in the previous experiments. The drops were allowed to dry and the plates were incubated at 22° C.

To obtain quantitative recovery of the cells, a flamed cork borer was used to cut plugs in the agar, each plug encompassing the area of a drop. Each plug was then suspended in 5.0 cc. salt solution and thoroughly triturated. Control experiments indicated that between 90 and 100% of the cells may be recovered from the plugs in this manner. Direct counts were made on each of the suspensions and appropriate dilutions were plated in quadruplicate with *A. aerogenes* according to the procedure given in the Methods section for clonally isolated platings. After incubation, total and differential plaque counts were made.

The plugs were collected at intervals until the beginning of aggregation. The drops prepared from mixture I showed evidence of aggregation at 12–14 hours. The cells in mixture II aggregated at 20–23 hours. Samples could not be taken

after these times because of excessive clumping of the cells and adherence to the agar.

Figure 4 illustrates the total population counts from four experiments. It will be noted that the viable counts cover the entire span between the deposition of the cells and the onset of aggregation. Each point represents the mean of counts obtained from four to eight plugs (16 to 32 plates). It is clear that no significant increase in cell number occurred on the aggregation plates. The mean number of plaques/plate was 59.2 with a variance of 109. The direct cell counts remained constant as well. The fact that extensive proliferation does not occur makes supposition (a) untenable.

Table I summarizes the results obtained from differential plaque counts. Neither mixture I nor II showed a significant change in the proportion of Nc-4 cells between zero time and the onset of aggregation. It must be concluded that assumption (b) is invalid.

TABLE I

The proportion of wild type cells in the mixed populations during incubation on minimal agar

Mixture	Time in hours after deposition	Total no. plaques	No. wild type plaques	% Wild type
I	2	1103	101	9.2
	4.5	1198	93	7.8
	8.5	347	31	9.0
	11	665	54	8.1
II	1–2	1019	15	1.5
	4.5	1096	16	1.5
	8.5	340	5	1.5
	11 –13.5	949	16	1.7
	14.5–16.5	501	10	2.0
	17.5–23.5	2095	34	1.6

Replicate population samples were collected at the stated intervals, suspended and plated. After incubation the plaques were surveyed and the proportions of wild type clones were determined.

Microscopic observation of aggregations of mixed populations has made supposition (c) highly unlikely. When the number of aggregateless cells is not overwhelmingly large, it may be seen that the entire area immediately surrounding the completed aggregate has been cleared of cells. Were the aggregateless individuals serving merely as conductors of the stimulus and not entering the aggregates, it would be impossible for every cell near the aggregate to have been removed since the ratio of aggregateless cells to wild type in such mixtures can be as high as 50:1. Moreover, visual comparison of aggregative patterns produced by control suspensions of the wild type alone and in conjunction with aggregateless cells revealed that the latter were far more extensive than the former.

In the absence of any other interpretations, one must conclude that the aggregateless cells do indeed enter into the aggregations evoked by wild type initiators. In this connection, the results of Pfützner-Eckert (1950) are of interest. Myx-

amoebae of the strain incapable of fruiting were shown to orient around and stream toward wild type pseudoplasmodia which had been placed in their midst.

E. The phenotypic composition of the sori from mixtures of wild type and agg-53

Mixtures of wild type and agg-53 were prepared in the usual manner and dispensed on minimal agar. The following aliquot volumes and proportions were employed:

Mixture	Vol. of aliquots delivered	No. Nc-4	No. Agg-53	Ratio Nc-4:Agg-53
1	0.01 cc.	6.0×10^3	3.0×10^5	1:50
2	0.02 cc.	6.0×10^3	5.0×10^5	1:83
3	0.04 cc.	6.0×10^3	8.4×10^5	1:139
4	0.08 cc.	6.0×10^3	1.4×10^6	1:232

In this manner a constant number of wild type cells was distributed over different surface areas while the variation in numbers of agg-53 provided constant population density. The particular concentrations of agg-53 were chosen on the basis of experiments (Sussman and Noël, 1952) from which the relation between the volume of aliquot and the surface area covered had been determined.

After the plates had been incubated for 36 hours, approximately 6 aggregative centers/aliquot had been formed in each of the mixtures. Single spore masses were picked and plated with bacteria. After four days incubation, the plaques were examined. The approximate numbers of plaques surveyed for the four mixtures cited above were, respectively, 1430, 1000, 350 and 204. In every case, not a single agg-53 plaque was observed. All produced normal fruiting bodies.

These results would appear to indicate that aggregateless cells do not enter into the aggregates initiated by the wild type. However, in view of the results described in the previous section, this is not considered to be a likely possibility. Two additional explanations can be offered. First, it may be presumed that only the wild type cells within the mixed aggregates eventually become spores. Second, one might suppose that any aggregateless cells which enter the aggregates either spontaneously revert or are transformed to wild type. The implications which the latter possibility possesses both for the genetic nature of the aggregateless variant and for cellular interactions during the developmental process would seem to demand that a distinction between these interpretations be attempted.

Discussion

The information at present available makes it possible to formulate a description, albeit tentative and incomplete, of the myxamoeboid population undergoing aggregation. During the stationary growth phase, the wild type population may be divided into at least two phenotypic classes. These are the initiator cells, each of which can evoke the formation of an aggregative center, and the responding cells, which can proceed to aggregate only upon reception of the stimulus imposed by an initiator cell.

Two independently derived sets of data indicate that only a small proportion of the cells possesses initiating capacity under the conditions of test. The precise proportion of initiators in *D. discoideum* is in doubt. An examination of center

formation by individuals of strain Nc-4 had previously indicated that there exists one initiator cell per 2110 myxamoebae (Sussman and Noël, 1952). But the results obtained from mixtures of wild type with Nc-4 agg-53A indicate a ratio of one initiator per 1720 cells and a corresponding analysis employing Nc-4 agg-53 as the test system suggests a value of one initiator per 980 cells. The discrepancy between the first two values is probably significant, that between the first or second and the last is certainly so.

A tentative explanation of these differences, which we favor currently as a working hypothesis, rests upon two assumptions: (a) There is a distribution of initiating ability in the wild type population, a few cells possessing a very high order, others inferior orders. These inequalities may stem from differences in a particular biosynthetic capacity which is associated with the process of initiation. (b) The ability of cells to respond to the initiating stimulus is a function of experimental conditions such as population density and depends also upon the strain of organisms employed.

From these suppositions, one might expect that for responding cells which possess a low level of sensitivity, only a portion of the potential initiators might have acquired sufficient initiating capacity to evoke the aggregative process. The greater the sensitivity of the responding cells, the higher would be the proportion of individuals which could be classed as initiators. In fact, were responding cells available which possessed extreme sensitivity, quite possibly the entire wild type population might be shown to have attained some degree of initiating ability.

On this basis, the previously cited discrepancies become understandable. When wild type individuals are mixed with aggregateless variants, the proportion of initiators will depend upon the sensitivity of the particular variant employed, hence the difference in the results obtained with agg-53 and agg-53A. Given aggregateless cells whose sensitivity approaches that of the responding cells in the wild type population, the proportion of initiators which appears in the mixture would be higher than when the wild type itself serves as the test system, even when the latter is at optimal population density. This stems from the fact that the density of responding cells in the former case can be made enormous and consequently even relatively small initiating capacities of some wild type cells would evoke a response. When wild type is employed alone, however, this concentration of responding cells cannot be attained. If one were to move the responding cells closer together, one would be simultaneously concentrating the initiator cells. The closer the proximity of these cells, the greater would be the opportunity for them to compete for responding cells, with the inevitable result that the maximal number of centers of which the population is capable would not appear and the apparent proportion of initiators would be decreased. Experimental evidence (Sussman and Noël, 1952) indicates that at population densities greater than 200–300 cells/mm.^{2} the decrease becomes significant and progressively larger. But this density is two orders of magnitude below that which can be achieved by addition of aggregateless cells.

While this is by no means the only interpretation which can be drawn, it offers readily testable inferences that are under examination at present. Its value lies in the fact that it offers an explanation of the genetic mechanism controlling the observed expression of cell heterogeneity. It suggests that the differences between initiator and responding cells may not be genic in nature but rather expressions of enzymatic adaptation against a constant genic background. That phenotypic altera-

tions involving relatively small segments of a population can be ascribed to non-genic variations in enzyme-forming capacity has been demonstrated in connection with the phenomenon of long-term adaptation in yeast (Spiegelman, Sussman and Pinska, 1950).

It is a pleasure to acknowledge the valuable assistance of Mrs. Min Zakarian in the performance of the experiments reported here.

Summary

1. A study has been made of the formation of aggregative centers by mixed populations of *D. discoideum* wild type and aggregateless variants. The latter are capable of responding to the aggregative stimulus but not of producing it. The following results were obtained:

(a) Under conditions where the wild type is too dispersed to aggregate alone, addition of aggregateless cells permits the formation of aggregative centers. When the number of centers so formed is plotted against the number of variant cells present, a saturation curve is obtained.
(b) For the case where the aggregateless cells are present in excess, the number of centers formed is directly proportional to the number of wild type added.
(c) Neither the total population nor the proportion of wild type cells changes significantly during the period of incubation prior to the onset of aggregation.

2. These findings demonstrate that the evocation of an aggregative center is accomplished by a single individual in the wild type population. These have been termed "initiator" cells. Under the conditions employed, the remainder of the wild type individuals and the aggregateless variants are the "responding cells" which can aggregate only in the presence of an initiator.

3. Estimates made of the proportion of initiators in the wild type population, using as test systems the wild type population itself as well as two aggregateless variants, range between one per 980 and 2110 individuals. The discrepancy is considered to be real and an explanation is offered.

Literature Cited

Bonner, J. T., 1944. A descriptive study of the development of the slime mold, *D. discoideum*. *Amer. J. Botany*, **31**: 175–182.

Bonner, J. T., 1947. Evidence for the formation of cell aggregates by chemotaxis in the development of the slime mold, *D. discoideum*. *J. Exp. Zool.*, **106**: 1–26.

Delerück, M., 1946. Bacterial viruses. *Biol. Rev.*, **21**: 30–40.

Pfützner-Eckert, R., 1950. Entwicklungsphysiologische Untersuchungen an *Distyostelium mucoroides* Brefeld. *Arch. f. Entw.*, **144**: 381–409.

Raper, K. B., 1940. Communal nature of the fruiting process in the Acrasiae. *Amer. J. Botany*, **27**: 436–448.

Spiegelman, S., R. R. Sussman and E. Pinska, 1950. On the cytoplasmic nature of long term adaptation in yeast. *Proc. Nat. Acad. Sci.*, **36**: 591–606.

Sussman, M., 1951. The origin of cellular heterogeneity in the slime molds, Dictyosteliaceae. *J. Exp. Zool.*, **118**: 407–418.

Sussman, M., and E. Noël, 1952. An analysis of the aggregation stage in the development of the slime molds, Dictyosteliaceae. I. The populational distribution of the capacity to initiate center formation. *Biol. Bull.*, **103**: 259–268.

INDEX

ABSTRACTS of papers presented at the Marine Biological Laboratory, 277.
Acrosome, reaction of to egg-water and other stimuli, 54.
ACTH, effect of on cleavage pattern of Arbacia ova, 305.
Action of DNase on cell nuclei, 319.
Activation of Nereis eggs by surface active extract of dead sperm, 305.
Adaptation to chemical stimulation of the tarsal receptors of the blowfly, 178.
Agglutination of fish erythrocytes by normal human sera, 328.
Aggregation stage in development of slime molds, analysis of, 259.
Aggregative center formation by mixtures of wild and aggregateless slime molds, 446.
Alcohol, ethyl, effects of on growth and respiration in Pelomyxa, 97.
ALFERT, MAX. Studies on basophilia of nucleic acids: The methyl green stainability of nucleic acids, 145.
Algae, flagella of, 74.
Alkaline sea water, reaction of acrosome to, 54.
Alkyl amines, halogenated, effects of on sea urchin egg division, 44.
ALLEE, W. C., and J. C. DICKINSON, JR. A contribution toward a knowledge of dominance in the smooth dogfish, Mustelus canis (Mitchill), 290.
ALLEN, ROBERT DAY. A visible response to stimulation in Spisula eggs, 283.
ALLEN, ROBERT DAY. The behavior of Spisula eggs with respect to potassium ions, 290.
Amaroecium, developmental history of, 226.
Ambystoma, methyl green stainability of nucleic acids in tissues of, 145.
Ameba, effects of ethyl alcohol on growth and respiration in, 97.
Amphibian gastrula explants, respiratory metabolism of, 407.
Analysis of the aggregation stage in the development of the slime molds, Dictyosteliaceae I. The populational distribution of the capacity to initiate aggregation, 259.
Analysis of aggregation stage in development of slime molds. II. Aggregative center formation by wild-type and aggregateless D. discoideum, 446.
ANDRUS, WILLIAM D. *See* GEORGE T. SCOTT, 308.
Antennal cuticle of honeybees, studies on, 201.
Annual Report of the Marine Biological Laboratory, 1.
Antigens, erythrocyte, of fish, 328.
Application of the most probable number method to determine heat sensitivity of protozoa, 364.
Arbacia, effects of halogenated alkyl amines on division of eggs of, 44.
Arbacia, electrical method for "sexing," 284.
ARMSTRONG, PHILIP B. Comparison of dye elimination in the developing liver and kidney (pronephros), 289.
ARMSTRONG, PHILIP B., and D. R. SHANKLIN. Facilitation of hydrogen ion passage across the chorion of Fundulus by the chlorides of calcium, potassium and sodium, 291.
ARMSTRONG, PHILIP B. *See* D. R. SHANKLIN, 295.
Arthropod muscle fibers, effects of calcium ion on, 421.
Arthropods, studies on cuticle of, 201.
Astacuran Crustacea, serological comparisons of, 433.
Atlantic Coast turbellarian fauna, notes on, 195.

BACILA, METRY, and R. R. RONKIN. On the presence of lactose in the hypobranchial gland of the snail, Busycon canaliculatum (L.), 296.
Bacillus subtilis, studies on poly-D-glutamic acid of, 291.
Balantidial infection in tadpoles as recorded by cinephotomicrography, 285.
BALL, E. G. *See* C. F. STRITTMATTER, 317.
BARTON, JAY, II. The action of DNase on cell nuclei, 319.
BARTON, JAY, II. Nucleoprotein complexes of sperm nuclei, 314.
Basophilia of nucleic acids, 145.
Bermudan Crustacea, bleeding and coagulation in, 395.
BERNSTEIN, G. S. Sperm agglutinins in the egg jelly of the frogs Rana pipiens Schreber and R. clamitans Latreille, 285.
Bleeding and coagulation in some Bermudan Crustacea, 395.
BLISS, DOROTHY E., and JOHN H. WELSH. The neurosecretory system of brachyuran Crustacea, 157.

Blood coagulation in certain decapod Crustacea, 316.
Blowfly, adaptation of tarsal receptors of to chemical stimulation, 178.
BOYCE, STEVE G. An ecological study of coastal plants on Cape Cod, 296.
BOYER, P. D. Studies on the distribution, potassium activation and mechanism of pyruvic phosphoferase, 314.
Brachyuran Crustacea, neurosecretory system of, 157.
Brain and prothoracic glands as endocrine system in the Cecropia silkworm, 120.
BRANDT, C. L. *See* A. C. GIESE, 336.
BROWN, FRANK A., JR., MURIEL I. SANDEEN and MILTON FINGERMAN. Modification of the tidal rhythm of Uca by tidal difference and by illumination, 297.
BROWN, FRANK A., JR., H. MARGUERITE WEBB and ROBERT C. GRAVES. A persistent tidal rhythm in the fiddler crab, Uca pugnax, 297.
BROWN, FRANK A., JR. *See* H. MARGUERITE WEBB, 310; MURIEL I. SANDEEN, 307.
Bugula, factors influencing metamorphosis of larvae of, 369.
BUTROS, JOSEPH M. On the relationship between cleavage block by respiratory inhibitors and viscosity changes in Ilyanassa eggs, 297.

CA-45, use of in measurement of rate of shell formation, 269.
Calcium ion, effect of on metamorphosis of Bugula larvae, 369.
Calcium, effects of on isolated arthropod muscle fibers, 421.
Callinectes sapidus, effects of calcium on isolated muscle fibers of, 421.
Cambarus, control of cement gland development in, 242.
Cancerous mice, attempt to influence survival time of, 282.
Carbohydrate metabolism, oxidative pathway of in E. coli, 287.
Cecropia silkworm, brain and prothoracic glands as endocrine system in, 120.
Cement gland development in the crayfish, 242.
CHAET, ALFRED B. The action of heparin preparations on cells and tissues, 281.
CHAET, A. B. *See* L. V. HEILBRUNN, 303.
Chaetopterus pergamentaceus, protoplasmic cortex of in relation to stimulation, 139.
Chemical stimulation, adaptation to of tarsal receptors of blowfly, 178.
Chlamydomonas, studies on flagella of, 74.
Chorion, Fundulus, osmotic behavior and anatomy of, 295.
CIERESZKO, LEON S. Studies on the nonsaponifiable fraction of lipid material from marine animals II., 315.
CIERESZKO, LEON S., and CHARLES R. CRANE, JR. Studies on the poly-D-glutamic acid of Bacillus subtilis NRRL-571, 296.
Ciona intestinalis, relation between water transport and food requirements of, 356.
CLARK, A. M., and C. J. MITCHELL. Effects of x-rays upon haploid and diploid embryos of Habrobracon, 170.
Cleavage block and viscosity changes in Ilyanassa eggs, 297.
Cleavage, effects of halogenated alkyl amines on, in sea urchin eggs, 44.
Cleavage initiation in frog eggs, 282.
Cleavage in marine eggs, initiation of by various agents, 139.
Cleavage synchrony in Arbacia, 288.
Coagulation and bleeding in Bermudan Crustacea, 395.
Cockroach, structure of peritrophic membrane of, 384.
Colchicine, effect of on melanophoral activity in excised frog skin, 312.
Cold, effect of on protoplasmic cortex rigidity, 139.
Colloidal changes in sea urchin egg homogenates, 293.
Colpidium colpoda, photoreactivation in, 336.
Comparative serology of astacuran Crustacea, 433.
CONNELLY, C. M. The oxygen consumption of squid nerve, 315.
Control of cement gland development in the crayfish, 242.
COOPER, O. *See* C. F. STRITTMATTER, 317.
CORNMAN, IVOR, and MARGARET EVANS CORNMAN. The effects of different halogenated alkyl amines on the division of sea urchin eggs, 44.
CORNMAN, IVOR, and EDWARD F. ROGERS. Chemical characteristics of rotenone antagonists as determined by effects on dividing sea urchin eggs, 298.
Cortex, protoplasmic, in relation to stimulation, 139.
COSTELLO, DONALD P., CATHERINE HENLEY and DONALD E. KENT. Effect of P-32 on mitosis in Chaetopterus eggs, 298.
COUILLARD, PIERRE. The fertilization inhibitor in the blood cells of Arbacia, 299.
Crab, effects of calcium on isolated muscle fibers of, 421.
Crago, response of distal retinal pigment of to changes in illumination, 307.
CRANE, CHARLES R., JR. *See* LEON S. CIERESZKO, 291.

Crassostrea virginica, measurement of rate of shell formation of, 269.
Crassostrea virginica, temperature requirements for maturation of gonads of, 80.
Crayfish, control of cement gland development in, 242.
Crayfish, serological comparisons of, 433.
Crustacea, astacuran, serological comparisons of, 433.
Crustacea, bleeding and coagulation, in, 395.
Crustacea, brachyuran, neurosecretory system of, 157.
CURRIER, JOANNE E. *See* MADELENE E. PIERCE, 306.
CUSHING, JOHN E., and LUCIAN SPRAGUE. The agglutination of fish erythrocytes by normal human sera, 328.
Cuticle, arthropod, studies on, 201.
Cymatogaster, agglutination of erythrocytes of by normal human sera, 328.
Cytochemistry of DNase, 319.

DAN, J. C. Studies on the acrosome. I. Reaction to egg-water and other stimuli, 54.
DAN, KATSUMA, SUSUMU ITO and DANIEL MAZIA. Study of the course of formation of the mitotic apparatus in Arbacia and Mactra by isolation techniques, 292.
DAN, KATSUMA. *See* D. MAZIA, 283.
Dark period, significance of in photoperiodic response of male birds, 345.
DAVIS, H. C. *See* V. L. LOOSANOFF, 80.
DAY, M. F. *See* E. H. MERCER, 384.
Desoxyribonucleic acid in nuclei of Arbacia eggs, 295.
Desoxyribonucleic acid in Arbacia eggs determined by isotope dilution, 294.
DETHIER, V. G. Adaptation to chemical stimulation of the tarsal receptors of the blowfly, 178.
Development of Cecropia silkworm adult as function of brain and prothoracic gland regulation, 120.
Development of cement gland in crayfish, 242.
Development of slime molds, analysis of aggregation stage in. I., 259.
Development of slime molds, analysis of aggregation stage in. II., 446.
Developmental history of Amaroecium constellatum. III. Metamorphosis, 226.
Diapause, brain and prothoracic glands as endocrine system in, 120.
DICKINSON, J. C., JR. *See* W. C. ALLEE, 290.
Dictyosteliaceae, analysis of aggregation stage in development of, 259.
Dictyostelium discoideum, aggregative center formation by mixtures of wild and aggregateless, 446.
Diploid Habrobracon embryos, effect of x-rays on, 170.
DI STEFANO, HENRY, and DANIEL MAZIA. The role of the midpiece in the incorporation of P-32 into the ribonucleic acid of Arbacia spermatozoa, 299.
DNase, action of on cell nuclei, 319.
Drosophila, influence of temperature on synapsis in hybrid salivary glands of, 67.
DUNN, ARNOLD. *See* L. V. HEILBRUNN, 33.
Dye elimination in developing liver and kidney, 289.

ECOLOGICAL study of coastal plants on Cape Cod, 296.
Effects of calcium on isolated arthropod muscle fibers, 421.
Effects of different halogenated alkyl amines on sea urchin egg division, 44.
Effects of ethyl alcohol on growth and respiration in Pelomyxa, 97.
Effects of x-rays upon haploid and diploid embryos of Habrobracon, 170.
Egg inclusions, electron microscopy of, 294.
Egg, protoplasmic cortex of in relation to stimulation, 139.
Egg surfaces, electron microscopy of, 293.
Egg-water, reaction of acrosome to, 54.
Eggs, effect of halogenated alkyl amines on division of, 44.
EICHEL, BERTRAM, and W. W. WAINIO. Comparison of the action of sodium adipate and sodium malonate on succinic dehydrogenase activity, 300.
EICHEL, BERTRAM. *See* PHILIP PERSON, 287.
EICHEL, HERBERT J., and JAY S. ROTH. Respiration and enzyme studies on x-irradiated Tetrahymena, 300.
Electron microscope study of sea urchin sperm, 54.
Electron microscopy of arthropod cuticle, 201.
Electron microscopy of insect peritrophic membrane, 384.
ELLIOTT, A. M., and D. F. GRUCHY. The occurrence of mating types in Tetrahymena, 301.
ELLIOTT, A. M., J. F. HOGG, J. V. SLATER and C. WU. Improved culture conditions for Tetrahymena, 301.
Embryos, Habrobracon, effects of x-rays on, 170.
Embryos, respiratory metabolism of, 407.
Endocrine system, brain and prothoracic gland as in Cecropia silkworm, 120.
ENGELS, WILLIAM L. *See* CHARLES E. JENNER, 345.
Eosin, effect of on metamorphosis of Bugula larvae, 369.

Erythrocytes, fish, agglutination of by normal human sera, 328.
Ethyl alcohol, effects on growth and respiration in Pelomyxa, 97.
EVANS, FREDERICK R., and ROBERT C. PENDLETON. A study of radiophosphate uptake in Paramecium multimicronucleatum, 190.
Excystation of apostome ciliate and relation of this process to molting of host crustacean, 295.
Explants, amphibian gastrula, respiratory metabolism of, 407.
Eyestalks, effects of removal of on rate of development of crayfish cement glands, 242.

FACTORS influencing metamorphosis of Bugula larvae, 369.
Fertilization inhibitor in blood cells of Arbacia, 299.
Fibrillar network of insect peritrophic membranes, 384.
FIGGE, FRANK H. J., and RALPH WICHTERMAN. The effects of hematoporphyrin and other substances upon x-ray sensitivity of Paramecium, 302.
FIGGE, FRANK H. J. *See* RALPH WICHTERMAN, 311.
Filter feeding invertebrates, relation between water transport and food requirements in, 356.
Fine structure of the peritrophic membranes of certain insects, 384.
FINGERMAN, MILTON. *See* FRANK A. BROWN, JR., 297; MURIEL I. SANDEEN, 307.
Fish erythrocytes, agglutination of by normal human sera, 328.
Flagella of algae, studies on, 74.
FLORKIN, MARCEL. Comparative studies on ectodermal scleroproteins, 315.
Food requirements and water transport in marine filter feeding invertebrates, 356.
Formation of insect peritrophic membranes, 384.
FOX, HOWARD. *See* ALLAN SCOTT, 288.
FRAENKEL, G. A function of the salivary glands in the larvae of Drosophila and other flies, 285.
FREEMAN, PAUL J. Some aspects of blood coagulation in certain decapod Crustacea, 316.
FROST, BETTY LU. *See* D. M. PACE, 97.
Fundulus heteroclitus, effect of centrifugation and low temperature on development of, 309.
Fundulus, neural tube extirpation in, 310.
Further notes on the turbellarian fauna of the Atlantic coast of the United States, 195.

GALLERIA mellonella, structure of peritrophic membrane of, 384.
Gastrulae, amphibian, respiratory metabolism of, 407.
Gecarcinus lateralis, neurosecretory system of, 157.
Genyonemus, agglutination of erythrocytes of by normal human sera, 328.
GIESE, A. C., C. L. BRANDT, R. IVERSON and P. H. WELLS. Photoreactivation in Colpidium colpoda, 336.
Globular proteins, recent results on structures of, 313.
Glycogen body as derivative of avian neural tube, 289.
Glycolytic activity in the swim bladder gland, 317.
GOLDSCHMIDT, ELISABETH. The influence of temperature on synapsis in hybrid salivary glands, 67.
GOLDSTEIN, LESTER. The mechanism of nuclear breakdown in the Chaetopterus egg, 292.
Gonads, oyster, temperature requirements for maturation of, 80.
Grasshopper, effects of calcium on isolated muscle fibers of, 421.
GRAVES, ROBERT C. *See* H. MARGUERITE WEBB, 310; FRANK A. BROWN, JR., 297.
GREGG, JOHN R. *See* NORMA ORNSTEIN, 407.
GROSCH, DANIEL S. The passage of vital stain from host to ectoparasitic wasp (Habrobracon), 302.
GROSS, PAUL R. An enzymatic study of yolk platelet lysis, 281.
GROSS, PAUL R. A study of colloidal changes in sea urchin egg homogenates, 293.
Growth, effects of ethyl alcohol on in Pelomyxa, 97.
Growth rate of oyster shells measured by use of Ca-45, 269.
GUTTMAN, RITA, ARNOLD M. KATZ and JOHN F. PERKINS, JR. Depolarization and contraction in smooth muscle on rapid cooling, 302.

HABROBRACON embryos, effects of x-rays on haploid and diploid, 170.
HALABAN, ATIDA. *See* L. V. HEILBRUNN, 282.
Halogenated alkyl amines, effects of on division of sea urchin eggs, 44.
Haploid embryos of Habrobracon, effects of x-rays on, 170.
HARVEY, ETHEL BROWNE. Electrical method of "sexing" Arbacia and obtaining small quantities of eggs, 284.
HAXO, FRANCIS. Carotenoid formation by mutant strains of Neurospora crassa, 286.

HAYWARD, HUGH R. *See* GEORGE T. SCOTT, 308.
Heat, effect of on protoplasmic rigidity, 139.
Heat sensitivity of protozoa, use of the most probable number method for determination of, 364.
HEILBRUNN, L. V., A. B. CHAET, ARNOLD DUNN and W. L. WILSON. A further study of the mitotic inhibitors in starfish extracts, 303.
HEILBRUNN, L. V., ATIDA HALABAN and W. L. WILSON. An attempt to influence the survival time of cancerous mice, 282.
HEILBRUNN, L. V. *See* W. L. WILSON, 139.
HEMPLING, H. G. K and Na content in dogfish leucocytes, 303.
HENLEY, CATHERINE. *See* DONALD P. COSTELLO, 298.
Heparin preparations, action of on cells and tissues, 281.
HILLIER, J., A. I. LANSING and T. B. ROSENTHAL. Electron microscopy of some marine egg surfaces, 293.
HILLIER, J. *See* A. I. LANSING, 294.
HOFFMAN, JOSEPH F. The action of uranyl salts on the erythrocyte surface, 303.
HOGG, J. F. *See* A. M. ELLIOTT, 301.
Homarus americanus, effects of calcium on isolated muscle fibers of, 421.
Honeybees, antennal cuticle of, 201.
Hormone, crustacean eyestalk, effect of on melanophore activity in excised frog skin, 312.
Hydrogen ion passage across Fundulus chorion, facilitation of by chlorides of calcium, potassium and sodium, 291.
Hormones in Cecropia silkworm, 120.
Hybrid Drosophila, influence of temperature on synapsis in salivary glands of, 67.
Hydra, regulation of spontaneous structural anomalies in, 104.
HYMAN, LIBBIE H. Further notes on the turbellarian fauna of the Atlantic Coast of the United States, 195.
Hypobranchial gland of Busycon, presence of lactose in, 296.
Hypobranchial mucus of Busycon, fibrous ultrastructure in, 306.

INFLUENCE of temperature on synapsis in hybrid salivary glands, 67.
INOUÉ, S. Effect of temperature on the birefringence of the mitotic spindle, 316.
Insect diapause, brain and prothoracic glands as endocrine system in, 120.
Insects, structure of peritrophic membranes of, 384.
Intercerebralis-cardiacum-allatum system of insects, further studies on, 284.
Invertebrates, marine filter feeding, relation between water transport and food requirements in, 356.
Iodoacetate effect, prevention of on sodium and potassium content of Ulva by photosynthesis, 308.
Isotopes, use of in predation experiments with Paramecium, 190.
ITO, SUSUMU. *See* J. R. SHAVER, 309; KATSUMA DAN, 292.
IVERSON, R. *See* A. C. GIESE, 336.

JENNER, CHARLES E., and WILLIAM L. ENGELS. The significance of the dark period in the photoperiodic response of male juncos and white-throated sparrows, 345.
JODREY, LOUISE H. *See* KARL M. WILBUR, 269.
JOHNSON, WILLIAM H., and JOHN F. PERKINS, JR. Studies on the visco-elastic properties of the anterior byssal retractor muscle of Mytilus edulis, 286.
JOHNSON, WILLIAM H., JOHN F. PERKINS, JR., and ARNOLD M. KATZ. The frequency dependence of the mechanical properties of smooth muscles, 293.
JØRGENSEN, C. BARKER. On the relation between water transport and food requirements in some marine filter feeding invertebrates, 356.
Juncos, significance of dark period in photoperiodic response of, 345.

KATZ, ARNOLD M. *See* RITA GUTTMAN, 302; WILLIAM H. JOHNSON, 293.
KENT, DONALD E. *See* DONALD P. COSTELLO, 298.
KING, JOHN W. On the reproductive rate of two algal species after treatment with growth-affecting substances, 304.
KOHN, ROBERT R. The influence of pituitary posterior lobe preparations on the action of phenolases in vitro, 304.
KOHN, ROBERT R. *See* PAUL A. WRIGHT, 312.

LANSING, A. I., J. HILLIER and T. B. ROSENTHAL. Electron microscopy of some marine egg inclusions, 294.
LANSING, A. I. *See* J. HILLIER, 293.
Larvae, Bugula, factors influencing metamorphosis of, 369.
LEONE, CHARLES A. *See* CARLON W. PRYOR, 433.
Lepidoptera, brain and prothoracic glands as an endocrine system in, 120.
Leucocytes, dogfish, K and Na content of, 303.

LEWIN, RALPH A. Studies on the flagella of algae. I. General observations on Chlamydomonas moewusii Gerloff, 74.
Light, effect of on development of crayfish cement glands, 242.
Light intensity and temperature, influence of on chromatophores of Palaemonetes, 310.
Lipid material, nonsaponifiable, studies on in marine animals, 315.
Locusta migratoria, structure of peritrophic membrane of, 384.
LOEFER, JOHN B., and ROY B. MEFFERD, JR. Application of the most probable number method to determine the heat sensitivity of protozoa, 364.
LOOSANOFF, VICTOR L., and H. C. DAVIS. Temperature requirements for maturation of gonads of northern oysters, 80.
LYNCH, WILLIAM F. Factors influencing metamorphosis of Bugula larvae, 369.
Lytechinus variegatus, effects of halogenated alkyl amines on division of eggs of, 44.

MAGNESIUM ion, effect of on metamorphosis of Bugula larvae, 369.
Marine filter feeding invertebrates, relation between water transport and food requirements in, 356.
MARSHAK, A., and C. MARSHAK. Desoxyribonucleic acid in the nuclei of Arbacia eggs, 294.
MARSHAK, A., and C. MARSHAK. Desoxyribonucleic acid in Arbacia eggs determined by isotope dilution, 295.
Mating behavior in Chlamydomonas moewusii, 74.
Mating types in Tetrahymena, 301.
Maturation of oyster gonads, temperature requirements for, 80.
MAZIA, D., and KATSUMA DAN. The isolation and chemical characterization of the mitotic apparatus of dividing cells, 283.
MAZIA, DANIEL. *See* KATSUMA DAN, 293; HENRY DI STEFANO, 299.
Measurement of rate of shell formation using Ca-45, 269.
MEFFERD, ROY B., JR. *See* JOHN B. LOEFER, 364.
Membranes, peritrophic, fine structure of, 384.
MENKIN, VALY. Effect and significance of various steroids and of ACTH (corticotropin) on the cleavage pattern of Arbacia ova, 305.
MERCER, E. H., and M. F. DAY. The fine structure of the peritrophic membrane of certain insects, 384.
Metabolism of amphibian gastrula explants, 407.
Metamorphosis of Amaroecium, 226.
Metamorphosis of Bugula larvae, factors influencing, 369.
Metamorphosis, role of brain and prothoracic glands in, in Cecropia silkworm, 120.
Method for determining heat sensitivity in protozoa, 364.
Methyl green stainability of nucleic acids, 145.
Methylene blue, effect of on metamorphosis of Bugula larvae, 369.
METZ, CHARLES B. *See* DOROTHY PITELKA, 282.
Micelle orientation in arthropod cuticle, 201.
Midpiece, role of in incorporation of P-32 into ribonucleic acid of Arbacia sperm, 299.
MITCHELL, C. J. *See* A. M. CLARK, 170.
Mitosis, effects of halogenated alkyl amines on in sea urchin eggs, 44.
Mitosis, effects of P-32 on in Chaetopterus eggs, 298.
Mitotic apparatus, formation of in Arbacia and Mactra as studied by isolation techniques, 292.
Mitotic inhibitor in starfish extracts, 303.
Molgula manhattensis, relation between water transport and food requirements of, 356.
MORRISON, KATHERINE C. *See* PETER R. MORRISON, 395.
MORRISON, PETER R., and KATHERINE C. MORRISON. Bleeding and coagulation in some Bermudan Crustacea, 395.
Most probable number method, application of to determine heat sensitivity of protozoa, 364.
Mouse tissues, methyl green stainability of nucleic acids in, 145.
Muscle fibers, arthropod, effects of calcium on, 421.
Myxamoebae, analysis of aggregation stage in development of, 259.

NEURAL tube extirpation in Fundulus, 310.
Neurohypophysis of elasmobranch fishes, 284.
"Neuromotor apparatus" of Paramecium, 282.
Neurosecretory system of brachyuran Crustacea, 157.
Neurospora crassa, carotenoid formation by mutant strains of, 286.
Neutral red, effect of on metamorphosis of Bugula larvae, 369.
NICHOL, CHARLES A. Studies on the distribution of vitamin B-12, 317.
Nitrogen mustards, effects of on division of sea urchin eggs, 44.
NOËL, ELIZABETH. *See* M. SUSSMAN, 259.
Nomenclature of turbellarians, 195.
Nuclear breakdown, mechanism of in Chaetopterus egg, 292.
Nuclei, action of DNase on, 319.

Nucleic acids, methyl green stainability of, 145.
Nucleoprotein complexes of sperm nuclei, 314.

ONION root tip nuclei, methyl green stainability of nucleic acids in, 145.
ORNSTEIN, NORMA, and JOHN R. GREGG. Respiratory metabolism of amphibian gastrulae explants, 407.
OSTERHOUT, W. J. V. Activation of eggs of Nereis limbata by a surface active extract of dead sperm, 305.
OSTERHOUT, W. J. V. Reversible contraction of protoplasmic structures by changes in pH values, 306.
Oxalate, use of in prevention of clotting reaction in calcium-treated arthropod muscle fibers, 421.
Oysters, measurement of rate of shell formation of, 269.
Oysters, relation between water transport and food requirements of, 356.
Oysters, studies on growth and ecology of, 306.
Oysters, temperature requirements for maturation of gonads of, 80.

P-32 uptake in P. multimicronucleatum, 190.
PACE, D. M., and BETTY LU FROST. The effects of ethyl alcohol on growth and respiration in Pelomyxa carolinensis, 97.
Parabiosis experiments in Cecropia silkworms, 120.
Paramecium, effects of hematoporphyrin and other substances upon x-ray sensitivity of, 302.
Paramecium, environmental factors concerned with lethality of x-rays in, 311.
Paramecium multimicronucleatum, P-32 uptake in, 190.
Pelmatohydra, regulation of spontaneous structural anomalies in, 104.
Pelomyxa carolinensis, effects of ethyl alcohol on growth and respiration of, 97.
PENDLETON, ROBERT C. *See* FREDERICK R. EVANS, 190.
Periplaneta americana, structure of peritrophic membrane of, 384.
Peritrophic membranes of insects, fine structure of, 384.
PERKINS, JOHN F., JR. *See* RITA GUTTMAN, 302; WILLIAM H. JOHNSON, 286, 293.
PERSON, PHILIP, W. W. WAINIO and B. EICHEL. The separation and partial identification of the prosthetic groups of cytochrome oxidase and cytochrome b, 287.
pH, effect of on "resting" Tetrahymena, 310.
Phormia regina, adaptation of tarsal receptors of to chemical stimulation, 178.
Photomicroscopy with Polaroid Land camera, 308.
Photoperiodic response of male birds, significance of dark period in, 345.
Photoreactivation in Colpidium, 336.
Physiology of insect diapause. IV. The brain and prothoracic glands as an endocrine system in the Cecropia silkworm, 120.
PIERCE, MADELENE E., and JOANNE E. CURRIER. Further studies on the growth of oysters under different ecological conditions in Rand's Harbor, 306.
PITELKA, DOROTHY, and CHARLES B. METZ. The structure of the "neuromotor apparatus" of Paramecium as revealed by the electron microscope, 282.
Pituitary posterior lobe preparations, influence of on action of phenolases in vitro, 304.
Platysamia cecropia, brain and prothoracic glands as an endocrine system in, 120.
Ploidy, relation of to x-ray damage in Habrobracon embryos, 170.
Populational distribution of capacity to initiate aggregation in slime molds, 259.
Potassium exchange in Ulva, 308.
Potassium ion, effect of on metamorphosis of Bugula larvae, 369.
Potassium ions and Spisula eggs, 290.
Predation experiments using P-32 as "tag" for Paramecium, 190.
PROCTOR, NATHANIEL K. The effects of calcium on isolated arthropod muscle fibers, 421.
Prosthetic groups of cytochrome oxidase and cytochrome b, separation and partial identification of, 287.
Protein structure, bearing of new results in on cytological interpretations, 313.
Prothoracic glands and brain as endocrine system in Cecropia silkworm, 120.
Protoplasmic cortex in relation to stimulation, 139.
Protozoa, use of most probable number method to determine heat sensitivity of, 364.
PRYOR, CARLON W., and CHARLES A. LEONE. Serological comparisons of astacuran Crustacea, 433.
Pseudocentrotus, reaction of sperm acrosome of to egg-water and other stimuli, 54.
Pyruvic phosphoferase, studies on distribution, potassium activation and mechanism of, 314.

RADIOCALCIUM, use of in measurement of rate of shell formation, 269.
Radiophosphate, uptake of in Paramecium, 190.
Radiosensitivity of diploid and daploid Habrobracon embryos, 170.

Rana pipiens embryos, respiratory metabolism of, 407.
Rate of shell formation, measurement of using Ca-45, 269.
Receptors, tarsal, adaptation of to chemical stimulation in blowfly, 178.
Regulation of spontaneous structural anomalies in Pelmatohydra, 104.
Relation between water transport and food requirements in some marine filter feeding invertebrates, 356.
Reproductive rate of algae after treatment with growth-affecting substances, 304.
Respiration, effects of ethyl alcohol on, in Pelomyxa, 97.
Respiratory inhibitors, effect of on melanophoral activity of frog skin in vitro, 311.
Respiratory metabolism of amphibian gastrula explants, 407.
Reversible contraction of protoplasmic structures by changes in pH values, 306.
Ribonucleases of liver and kidney, comparative study of, 288.
RICHARDS, A. GLENN. Studies on arthropod cuticle. VIII. The antennal cuticle of honeybees with particular reference to the sense plates, 201.
Rigidity of protoplasmic cortex, effect of various agents on, 139.
ROGERS, EDWARD F. *See* IVOR CORNMAN, 298.
RONKIN, R. R. Fibrous ultrastructure in the hypobranchial mucus of Busycon, 306.
RONKIN, R. R. *See* METRY BACILA, 296.
ROOFE, PAUL G. Notes on the circulation in the yolk sac of the toad fish, Opsanus tau, 307.
ROSENTHAL, T. B. *See* A. I. LANSING, 293; J. HILLIER, 294.
Rotenone antagonists, chemical characteristics of, as determined by effects on dividing sea urchin eggs, 298.
ROTH, JAY S. A comparative study of the ribonucleases of liver and kidney, 288.
ROTH, JAY S. *See* HERBERT J. EICHEL, 300.

SABAL, JAMES. *See* PAUL A. WRIGHT, 312.

Salivary glands, function of in Drosophila and other flies, 285.
Salivary glands, influence of temperature on synapsis in, 67.
SANDEEN, MURIEL I., MILTON FINGERMAN and FRANK A. BROWN, JR. Responses of the distal retinal pigment of Crago septemspinosus to changes in illumination, 307.
SANDEEN, MURIEL, I. *See* FRANK A. BROWN, JR., 297.
SCHARRER, BERTA. Further studies of intercerebralis-cardiacum-allatum system of insects, 284.
SCHARRER, ERNST. The neurohypophysis of elasmobranch fishes, 284.
SCHECHTER, VICTOR. Survival of Spisula (Mactra) ova, 309.
Schistocerca americana, effects of calcium on isolated muscle fibers of, 421.
Scleroproteins, comparative studies on, 315.
SCOTT, ALLAN, and HOWARD FOX. Cleavage synchrony in individual embryos and in populations of embryos of Arbacia punctulata, 288.
SCOTT, DWIGHT B. MCNAIR. The oxidative pathway of carbohydrate metabolism in E. coli, 287.
SCOTT, SISTER FLORENCE MARIE. The developmental history of Amaroecium constellatum. III. Metamorphosis, 226.
SCOTT, GEORGE T., HUGH R. HAYWARD and WILLIAM D. ANDRUS. The prevention of the iodoacetate effect on the sodium and potassium content of Ulva lactuca by photosynthesis, 308.
SCOTT, GEORGE T., HUGH R. HAYWARD and WILLIAM D. ANDRUS. The rate and extent of exchange of K-42 with cellular K-39 in Ulva lactuca, 308.
Sea urchin eggs, effects of halogenated alkyl amines on division of, 44.
Sea urchin sperm, reaction of acrosome of to egg-water and other stimuli, 54.
Sense plate of honeybee antennal cuticle, 201.
Sera, human, agglutination of fish erythrocytes by, 328.
Serological comparisons of astacuran Crustacea, 433.
Sexing Arbacia, electrical method for, 284.
SHANKLIN, D. R. The technique of photomicroscopy with the Polaroid Land camera, 308.
SHANKLIN, D. R., and P. B. ARMSTRONG. The osmotic behavior and anatomy of the Fundulus chorion, 295.
SHANKLIN, D. R. *See* P. B. ARMSTRONG, 291.
SHAVER, J. R., and SUSUMU ITO. The effect of centrifugation and low temperature on development of Fundulus heteroclitus, 309.
SHAVER, J. R., S. SUBTELNY and A. WANIA. Studies on initiation of cleavage in frog eggs, 282.
Shell formation, studies on, 269.
Significance of the dark period in the photoperiodic response of male juncos and white-throated sparrows, 345.
Sinus gland of brachyuran Crustacea, histology of, 157.

SLATER, JOHN V. Effect of pH on "resting" Tetrahymena, 310.
SLATER, J. V. *See* A. M. ELLIOTT, 301.
Slime molds, analysis of aggregation stage in development of, I., 259.
Slime molds, analysis of aggregation stage in development of, II., 446.
Smooth muscle, depolarization and contraction in on rapid cooling, 302.
Smooth muscles, frequency dependence of mechanical properties of, 293.
Sodium ion, effect of on metamorphosis of Bugula larvae, 369.
Sparrows, significance of dark period in photoperiodic response of, 345.
Spawning of oysters, temperature requirements for, 80.
Species-specificity of fish erythrocyte antigens, 328.
SPEIDEL, CARL CASKEY. Balantidial infection in tadpoles as recorded by cine-photomicrography, 285.
Sperm, action of DNase on, 319.
Sperm agglutinins in egg jelly of Rana pipiens and R. clamitans, 285.
Spermatozoa, effects of egg-water and other stimuli on acrosome of, 54.
Spindle, isolation and chemical characterization of, 283.
Spisula (Mactra) ova, survival of, 309.
Spisula ova, visible response to stimulation in, 283.
SPRAGUE, LUCIAN. *See* JOHN E. CUSHING, 328.
Squid nerve, oxygen consumption of, 315.
STEPHENS, GROVER C. The control of cement gland development in the crayfish, Cambarus, 242.
Stimulation, adaptation to of tarsal receptors of blowfly, 178.
Stimulation, protoplasmic cortex in relation to, 139.
STRITTMATTER, C. F., E. G. BALL and O. COOPER. Glycolytic activity in the swim bladder gland, 317.
Strongylocentrotus, reaction of sperm acrosome of to egg-water and other stimuli, 54.
Structural anomalies, regulation of in Pelmatohydra, 104.
Structure of insect peritrophic membranes, 384.
Studies on arthropod cuticle. VIII., 201.
Studies on basophilia of nucleic acids: The methyl green stainability of nucleic acids, 145.
Studies on shell formation. I. Measurement of the rate of shell formation using Ca-45, 269.
Studies on the acrosome. I. Reaction to egg-water and other stimuli, 54.
Studies on the flagella of algae. I. General observations on Chlamydomonas moewusii Gerloff, 74.
SUBTELNY, S. *See* J. R. SHAVER, 282.
Succinic dehydrogenase, comparison of action of sodium adipate and sodium malonate on, 300.
SUSSMAN, M. An analysis of the aggregation stage in the development of the slime molds, Dictyosteliaceae. II. Aggregative center formation by mixtures of Dictyostelium discoideum wild type and aggregateless variants, 446.
SUSSMAN, M., and ELIZABETH NOËL. An analysis of the aggregation stage in the development of the slime molds, Dictyosteliaceae. I. The populational distribution of the capacity to initiate aggregation, 259.
Synapsis, influence of temperature on in hybrid salivary glands, 67.

—

TARSAL receptors, adaptation of to chemical stimulation in blowfly, 178.
Temperature, influence of on synapsis in hybrid salivary glands, 67.
Temperature requirements for maturation of gonads of northern oysters, 80.
Temperature sensitivity of protozoa, use of most probable number method for determination of, 364.
Testis size in birds as a function of light, 345.
Tetrahymena, improved culture conditions for, 301.
Tetrahymena, respiration and enzyme studies on x-irradiated, 300.
Tetrahymena, use of most probable number method for determination of heat sensitivity of, 364.
Thyroid gland, chick, radioiodine uptake by, 289.
TRAGER, WILLIAM. The relation between the excystation of an apostome ciliate (Gymnodinioides sp.) and the molting of its crustacean host (Uca pugnax), 295.
Tripneustes, effects of halogenated alkyl amines on division of eggs of, 44.
Turbellarian fauna of Atlantic Coast of United States, notes on, 195.
TURNER, C. L. The regulation of spontaneous structural anomalies in Pelmatohydra oligactis, 104.

UCA, modification of tidal rhythm of by tidal differences and by illumination, 297.
Uca, persistent tidal rhythm in, 297.
Ultraviolet treatment of Colpidium, 336.
Uranyl salts, action of on erythrocyte surface, 303.

Urethan, synergism of with alkyl amines in effects on sea urchin egg division, 44.

VARIANTS of D. discoideum, 446.

Visco-elastic properties of anterior byssal retractor muscle of Mytilus, 286.

Vital dyes, effects of on metamorphosis of Bugula larvae, 369.

Vital stain, passage of from host to ectoparasitic wasp (Habrobracon), 302.

Vitamin B-12, studies on distribution of, 317.

WAINIO, W. W. *See* PHILIP PERSON, 287; BERTRAM EICHEL, 300.

WANIA, A. *See* J. R. SHAVER, 282.

Water transport and food requirements in marine filter feeding invertebrates, 356.

WATTERSON, RAY L. The glycogen body as a derivative of the avian neural tube, 289.

WATTERSON, RAY L. Neural tube extirpation in Fundulus heteroclitus and resultant neural arch defects, 310.

WEBB, H. MARGUERITE, F. A. BROWN, JR., and ROBERT C. GRAVES. The influence of light intensity and temperature on chromatophores of Palaemonetes vulgaris, 310.

WEBB, H. MARGUERITE. *See* FRANK A. BROWN, JR., 297.

WELLS, P. H. *See* A. C. GIESE, 336.

WELSH, JOHN H. *See* DOROTHY E. BLISS, 157.

WICHTERMAN, RALPH, and FRANK H. J. FIGGE. Environmental factors concerned with the lethality of x-rays in Paramecium, 311.

WICHTERMAN, RALPH. *See* FRANK H. J. FIGGE, 302.

WILBUR, KARL M., and LOUISE H. JODREY. Studies on shell formation. I. Measurement of the rate of shell formation using Ca-45, 269.

WILLIAMS, CARROLL, M. Physiology of insect diapause. IV. The brain and prothoracic glands as an endocrine system in the Cecropia silkworm, 120.

WILSON, W. L., and L. V. HEILBRUNN. The protoplasmic cortex in relation to stimulation, 139.

WILSON, WALTER L. *See* L. V. HEILBRUNN, 282; 303.

WOLLMAN, SEYMOUR, and EDGAR ZWILLING. Radioiodine uptake in the chick embryo thyroid gland, 289.

WRIGHT, PAUL A. Effect of respiratory inhibitors on melanophoral activity of frog skin in vitro, 311.

WRIGHT, PAUL, A., and ROBERT R. KOHN. Effect of crustacean eyestalk hormone on melanophore activity in excised frog skin, 312.

WRIGHT, PAUL A., and JAMES SABAL. Effect of colchicine on melanophoral activity in excised frog skin (Rana pipiens), 312.

WRINCH, DOROTHY. The bearing of new results in protein structure on cytological interpretations, 313.

WRINCH, DOROTHY. Recent results regarding the structure of the globular proteins, 313.

WU, C. *See* A. M. ELLIOTT, 301.

X-RAYS, effects of on haploid and diploid embryos of Habrobracon, 170.

YOLK platelet lysis, enzymatic study of, 281.

Yolk sac circulation in the toadfish, 307.

ZONOTRICHIA albicollis, significance of dark period in photoperiodic response of, 345.

ZWILLING, EDGAR. *See* SEYMOUR H. WOLLMAN, 289.

Volume 103 Number 3

DEC 2 1952
SCIENCE
LIBRARY

THE BIOLOGICAL BULLETIN

PUBLISHED BY
THE MARINE BIOLOGICAL LABORATORY

Editorial Board

W. C. ALLEE, University of Florida
L. R. BLINKS, Stanford University
DANIEL MAZIA, University of California
A. K. PARPART, Princeton University
C. LADD PROSSER, University of Illinois
A. GLENN RICHARDS, University of Minnesota
F. SCHRADER, Columbia University
H. B. STEINBACH, University of Minnesota
WM. RANDOLPH TAYLOR, University of Michigan
ALBERT TYLER, California Institute of Technology
DOUGLAS WHITAKER, Stanford University
RALPH WICHTERMAN, Temple University

DONALD P. COSTELLO, University of North Carolina
Managing Editor

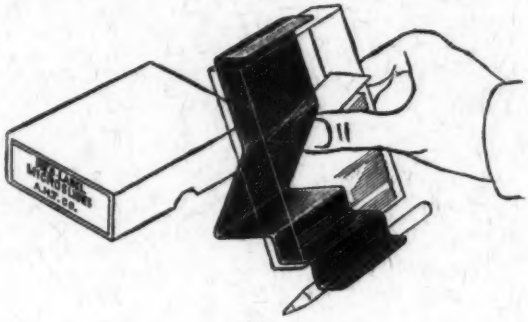
DECEMBER, 1952

Printed and Issued by
LANCASTER PRESS, Inc.
PRINCE & LEMON STS.
LANCASTER, PA.

A. H. T. CO. SPECIFICATION

STAND FOR MICRO SLIDE BOXES

For tilting a half-gross box of Micro Slides so that the Slides can be removed singly without touching the flat surfaces



7053.

MICRO SLIDE BOX STAND, A.H.T. Co. Specification. For tilting a half-gross box of 3 x 1-inch, A.H.T. Co. Specification, Red Label Micro Slides (as described on page 889 of our general catalogue) on end so that the slides can be removed singly, as required, without touching the flat surfaces. The inclined position of the stack of slides in the stand greatly simplifies handling in routine use. Boxes for A.H.T. Co. Red Label 3 x 1-inch slides have recently been re-designed so that the upper edges of the slides extend approximately 1/8-inch above the carton sides to permit ready removal by grasping the ends.

Stand is made of sheet aluminum, approximately 65 mm wide, and fits snugly over the ends of the carton and is shaped to provide a stable base, 75 mm deep, with a V-shaped pencil rest in convenient position. Box lid can be replaced, for protecting the slides from dust, without removal of the box from the stand, by taking the complete assembly in one hand and, with the other hand, fitting the upper end of the lid first and then gently depressing the pencil rest as the lid is seated.

7053. Stand for Micro Slide Box, A.H.T. Co. Specification, as above described, of aluminum with etched finish..... **.70**
10% discount in lots of 12.

NOTE—Stand can be used with almost any Slide carton which is approximately 4 inches long. Size 3 x 1½ or 3 x 2-inch slides can be elevated above the edges of such cartons by inserting layers of cardboard beneath them.

ARTHUR H. THOMAS COMPANY

LABORATORY APPARATUS AND REAGENTS

WEST WASHINGTON SQUARE
PHILADELPHIA 5, PA.

Teletype Services: Western Union WUX and Bell System PH-72

BIOLOGICAL ABSTRACTS

COVERS THE WORLD'S BIOLOGICAL LITERATURE

How do you keep abreast of the literature in your field? No individual possibly could accumulate and read all of the biological contributions in the original—yet some relatively obscure journal might publish a revealing paper on the very subject in which you are most interested.

Biological Abstracts now publishes concise, informative abridgments of all the significant contributions from more than 2,500 journals. As well as the complete edition, it also is published in nine low-priced sectional editions which are specially designed for individuals who are interested only in one or more closely related fields.

Production costs have increased to such an extent that the active support of all biologists is needed to maintain this important service. Write for full details and a sample copy of the sectional edition covering your field.

BIOLOGICAL ABSTRACTS
UNIVERSITY OF PENNSYLVANIA
PHILADELPHIA 4, PA.

MICROFILM SERVICE

The Library of The Marine Biological Laboratory can supply microfilms of material from periodicals included in its list. Requests should include the title of the paper, the author, periodical, volume and date of publication.

Rates are as follows: $1.00 for papers up to 50 pages, and $.10 for each additional 10 pages or fraction thereof.

LANCASTER PRESS, Inc.

LANCASTER, PA.

THE EXPERIENCE we have gained from printing some sixty educational publications has fitted us to meet the standards of customers who demand the best.

We shall be happy to have workers at the MARINE BIOLOGICAL LABORATORY write for estimates on journals or monographs. Our prices are moderate.

INSTRUCTIONS TO AUTHORS

The Biological Bulletin accepts papers on a variety of subjects of biological interest. In general, however, review papers (except those written at the specific invitation of the Editorial Board), short preliminary notes and papers which describe only a new technique or method without presenting substantial quantities of data resulting from the use of the new method cannot be accepted for publication. A paper will usually appear within three months of the date of its acceptance.

The Editorial Board requests that manuscripts conform to the requirements set below; those manuscripts which do not conform will be returned to authors for correction before they are refereed by the Board.

1. ***Manuscripts.*** Manuscripts must be typed in double spacing (*including* figure legends, foot-notes, bibliography, etc.) on one side of 16- or 20-lb. bond paper, 8½ by 11 inches. They should be carefully proof-read before being submitted and all typographical errors corrected legibly in black ink. Pages should be numbered. A left-hand margin of at least 1½ inches should be allowed.

2. ***Tables, Foot-Notes, Figure Legends, etc.*** Tables should be typed on separate sheets and placed in correct sequence in the text. Because of the high cost of setting such material in type, authors are earnestly requested to limit tabular material as much as possible. Similarly, foot-notes to tables should be avoided wherever possible. If they are essential, they should be indicated by asterisks, daggers, etc., rather than by numbers. Foot-notes in the body of the text should also be avoided unless they are absolutely necessary, and the material incorporated into the text. Text foot-notes should be *numbered* consecutively and typed *double*-spaced on a separate sheet. Explanations of figures should be typed double-spaced and placed on separate sheets at the end of the paper.

3. A ***condensed title*** or running head of no more than 35 letters and spaces should be included.

4. ***Literature Cited.*** The list of papers cited should conform **exactly** to the style set in a recent issue of The Biological Bulletin; this list should be headed LITERATURE CITED, and typed *double*-spaced on separate pages.

5. ***Figures.*** The dimensions of the printed page, 5 by 7½ inches, should be kept in mind in preparing figures for publication. Illustrations should be large enough so that all details will be clear after appropriate reduction. Explanatory matter should be included in legends as far as possible, not lettered on the illustrations. Figures should be prepared for reproduction as line cuts or halftones; other methods will be used only at the author's expense. Figures to be reproduced as line cuts should be drawn in black ink on white paper, good quality tracing cloth or blue-lined coordinate paper; those to be reproduced as halftones should be mounted on Bristol Board, and any designating numbers or letters should be made directly on the figures. *All* figures should be numbered in consecutive order, with no distinction between text- and plate-figures. The author's name should appear on the reverse side of all figures, as well as the desired reduction.

6. ***Mailing.*** Manuscripts should be packed flat; large illustrations may be rolled in a mailing tube. **All illustrations larger than 8½ by 11 inches must be accompanied by photographic reproductions or tracings that may be folded to page size.**

Reprints. Authors will be furnished, free of charge, one hundred reprints without covers. Additional copies may be obtained at cost; approximate prices will be furnished by the Managing Editor upon request.

THE BIOLOGICAL BULLETIN

THE BIOLOGICAL BULLETIN is issued six times a year at the Lancaster Press, Inc., Prince and Lemon Streets, Lancaster, Pennsylvania.

Subscriptions and similar matter should be addressed to The Biological Bulletin, Marine Biological Laboratory, Woods Hole, Massachusetts. Agent for Great Britain: Wheldon and Wesley, Limited, 2, 3 and 4 Arthur Street, New Oxford Street, London, W. C. 2. Single numbers, $2.50. Subscription per volume (three issues), $6.00.

Communications relative to manuscripts should be sent to the Managing Editor, Marine Biological Laboratory, Woods Hole, Massachusetts, between June 15 and September 1, and to Dr. Donald P. Costello, Department of Zoology, University of North Carolina, Chapel Hill, North Carolina, during the remainder of the year.

Entered as second-class matter May 17, 1930, at the post office at Lancaster, Pa.
under the Act of August 24, 1912.

BIOLOGY MATERIALS

The Supply Department of the Marine Biological Laboratory has a complete stock of excellent plain preserved and injected materials, and would be pleased to quote prices on school needs.

PRESERVED SPECIMENS

for

Zoology, Botany, Embryology, and Comparative Anatomy

LIVING SPECIMENS

for

Zoology and Botany

including Protozoan and Drosophila Cultures, and Animals for Experimental and Laboratory Use.

MICROSCOPE SLIDES

for

Zoology, Botany, Embryology, Histology, Bacteriology, and Parasitology.

CATALOGUES SENT ON REQUEST

Supply Department

MARINE BIOLOGICAL LABORATORY

Woods Hole, Massachusetts

CONTENTS

Page

BARTON, JAY, II
The action of DNase on cell nuclei 319

CUSHING, JOHN E., AND LUCIAN SPRAGUE
The agglutination of fish erythrocytes by normal human sera 328

GIESE, A. C., C. L. BRANDT, R. IVERSON AND P. H. WELLS
Photoreactivation in Colpidium colpoda 336

JENNER, CHARLES E., AND WILLIAM L. ENGELS
The significance of the dark period in the photoperiodic response of male juncos and white-throated sparrows 345

JØRGENSEN, C. BARKER
On the relation between water transport and food requirements in some marine filter feeding invertebrates 356

LOEFER, JOHN B., AND ROY B. MEFFERD, JR.
Application of the most probable number method to determine heat sensitivity of protozoa 364

LYNCH, WILLIAM F.
Factors influencing metamorphosis of Bugula larvae 369

MERCER, E. H., AND M. F. DAY
The fine structure of the peritrophic membranes of certain insects 384

MORRISON, PETER R., AND KATHERINE C. MORRISON
Bleeding and coagulation in some Bermudan Crustacea 395

ORNSTEIN, NORMA, AND JOHN R. GREGG
Respiratory metabolism of amphibian gastrula explants 407

PROCTOR, NATHANIEL K.
The effects of calcium on isolated arthropod muscle fibers .. 421

PRYOR, CARLON W., AND CHARLES A. LEONE
Serological comparisons of astacuran Crustacea 433

SUSSMAN, M.
An analysis of the aggregation stage in the development of the slime molds, Dictyosteliaceae. II. Aggregative center formation by mixtures of Dictyostelium discoideum wild type and aggregateless variants 446